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EPIDEMIOLOGICAL MARKERS FOR *PSEUDOMONAS AERUGINOSA*

4 Change of O-antigen and Phage Sensitivity after Phage Infection in vitro *and* in vivo *of* Pseudomonas aeruginosa

TOM BERGAN and TORE MIDTVEDT

Kaptein W Wilhelmsen og Frues Bakteriologiske Institutt, Rikshospitalet, University of Oslo, Oslo, Norway

Bergan, T & Midtvedt, T Change of O-antigen and phage sensitivity after phage infection *in vitro* and *in vivo* of *Pseudomonas aeruginosa* Acta path. microbiol. scand. Sect. B, 83 1–9, 1975

A non-lysogenic strain of *Pseudomonas aeruginosa* maintained its phage sensitivity pattern and serogroup specificity unchanged for 10 weeks in ex-germfree, mono-contaminated rats before infection with phage. After infection with phage, phage conversions of serogroup specificity and lysotype were observed. With the same combination of bacterial and phage strains, the same serogroup was obtained *in vitro* and *in vivo* *In vitro* conversion occurred also to serogroups that were not detected *in vivo* Upon lyophilization, converted bacterial clones from the *in vivo* experiment lost their phage and simultaneously reverted to the original phage type and serogroup. These findings may have implications for the understanding of the degree of stability in epidemiological typing results for *P aeruginosa*

Key words O-antigen change phage sensitivity; *Pseudomonas aeruginosa* phage infection germ free life.

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Serial cultures of *Pseudomonas aeruginosa* from the same foci in the same patients occasionally show changes in phage type and serogroup (3) This may reflect alterations of the properties of the strains, or superinfection by new strains. *In vitro* *Liu* (13) has shown that lysogenization may change both the phage typing pattern and the serogroup of pseudomonas strains.

The purpose of the present investigation was to study serogroup changes after lysogenization of *P aeruginosa in vivo* in mono-contaminated gnotobiotic rats and to monitor stability of serogroup and phage type of faecal bacteria of animals.

MATERIALS AND METHODS

Bacterial and Bacteriophage Strains

As bacterial strain was used *P aeruginosa* 1C (from Dr *B. W Holloway* Clayton, Victoria, Australia) The 1C-strain was not lysogenic as evidenced by attempted UV-induction (*vide inf a*) It was initially cloned four times by spreading of individual colonies on blood agar before transfer to the germfree animals.

As bacteriophages two strains were selected to which the 1C-strain was susceptible, F116 (from Dr *Holloway*) and O1c (from Dr. *V L. Sutter* Los Angeles, California, U.S A.) The phage F116 on 1C (its propagating strain) produced opaque plaques of approximately 0.5 mm diameter the phage O1 plaques of 1–2 mm with a ca. 1 mm clear centre of complete lysis (cfr Fig. 1 in reference (1)) surrounded by a hazy halo. Upon

transfer to the rats, the phage suspension titres for F116 was 7×10^4 plaque forming units (PFU) per ml and for Olc 5×10^7 PFU

Animals

Four germfree CDF rats (Charles River Breeding Lab., Wilmington, Mass. U.S.A.) 40–50 days old were used in each of two *in vivo* experiments. The animals were kept in separate cages within the same isolator. The germfree techniques and the diet have been described previously (15)

Plan of Study

A. *In vitro* tests were initially performed to identify a suitable *P aeruginosa*-bacteriophage system to be studied *in vivo*. One bacterial strain and two phages were selected.

B. *In vivo* examinations were then performed twice on four germfree rats these were first monocontaminated by *P aeruginosa*. After colonization, multiple single colonies from faecal samples were examined weekly for 10 weeks to detect spontaneous changes in serogroup and phage type. Subsequently one phage, F116 was given to the monocontaminated animals, the stool flora was *examined repeatedly for changes in the epidemiological* types of the bacteria, followed, after three weeks when the phage typing pattern had stabilized, by superinfection with the other phage, C1c, to which the bacteria were still sensitive. In each phage typing, the IC-strain from a recently opened lyophilized ampoule was included for control.

Since no spontaneous changes in phage type or serogroup were revealed in the first group of animals (Experiment I) only one week elapsed between bacterial colonization and phage infection the second time (Experiment II)

Epidemiological Typing

Phage typing was done according to a previously described method (1) using the phages 73 F7 M6 Me13 113 F116, Px3 Clc, C4 C13 C21 H249 P10 VII, XVI, Z2 Z3 Z19 and Z20.

Serogrouping was carried out by the O-grouping *scheme of Habs* (8)

Lysogenization in vitro

Lysogenization *in vitro* was attempted in the following way

1 Double layer agar plates (medium described in (1)) with a top layer of 0 6 per cent Agar (Difco) were prepared with a suitable density of an over-night broth culture of the cloned IC-strain and phage (such that discrete plaques appeared)

The opaque zones of individual plaques developing after over-night incubation at 37° C were stabbed. The inoculating needle was then washed in sterile broth of the same composition as the agar (ex agar) and the broth incubated for 2 hours at 37 C. The culture was then inoculated on blood agar plates.

3 Single colonies were subsequently spread on agar three times successively to obtain a single-cell line.

4 Individual colonies were then examined for prophage, serogroup, and typing pattern.

Induction of Prophage

1 Colonies were picked and transferred to broth after three successive subcultures of single colonies to obtain pure single-cell clones.

2. UV-irradiation was carried out for 0 10 20, and 30 seconds on a thin layer of 5 ml broth in 9 cm diameter Petri-plates with Cromatolite Portable Ultraviolet Lamp (Hanovia Lamps) of 30 watts and 5A with the lamp 10 cm from the liquid surface. The broths were then incubated for 4 hours at 37 C, and centrifuged

4 Drops of the supernatants were applied on agar inoculated immediately before with the IC-*strain*.

5 Lytic areas were stabbed and the inoculating needle washed in tubes with 2 ml melted soft agar followed by plating with the *soft-agar overlay method* (1) to ensure that growth inhibition was caused by phage (and not by pyocine) This also enabled display of plaque morphology

6 The liberated phages were examined for lytic spectra on the propagating strains of the phage typing set. In the comparison of the phages lytic spectra, differences in one strong reaction were accepted (2)

Microbiological Procedures in the Axenic System

Before introduction of the bacterium, it had been grown over-night in glucose infusion broth. The undiluted cultures and phage suspensions were introduced into the isolator of the gnotobiotic rats in sealed peracetic acid sterilized glass ampoules and given to the animals in aliquots of 0.8 ml orally and *per rectum* within three hours of sealing the ampoules.

No discernible reaction, or change in the state of health of the rats was observed after bacterial colonization.

To isolate pseudomonas from the faeces, specimens from the rats were diluted in physiological saline and spread on lactose bromothymol blue agar and human blood agar to produce single colonies. Bacteria other than *P aeruginosa* were never seen.

TABLE 1 *Phage Sensitivity Pattern and Serogroup of Non-Lysogenised and Lysogenised Clones of* Pseudomonas aeruginosa *IC I fected In vitro by the Phages F116 and C1c Singly and in Succession*

Phage infection	Phage sensitivity pattern	Changes in phage pattern after exposure to phage relative to parent strain: Gains	Losses	Lysogenic	Serogroup	Number of corresponding clones
None	M6, Me13, 113 F116, Px3 C1c, H249			—	5	5
F116	M6, Me13 113 F116, Px3 C1c, H249			—	5	9
	C4 H249 Z2	C4 Z2	M6 Me13 113 F116 Px3 C1c	+	6	6
C1c	M6, Me13 113 F116, Px3, C1c, H249			—	5	15
F116 + C1c‡	M6, Me13, 113 F116, C1c, H249	C1c	Px3	—	5	12
	M6, M 13		113 F116 Px3 C1c, H249	+	9	2
	M6 Z20	Z20	Me13 113 F116 Px3 C1c, H249	+	9	1
C1c + F116	C4 H249 Z2	C4 Z2	M6, Me13 113 F116, Px3 C1c	+	6	15

‡ "F116 + C1c" denotes that IC was first superinfected with F116; after subcultivation (to separate clones) followed C1c. The clone used for C1c infection was nonlysogenic and of serogroup O 5 since the lysogenic variants did not exhibit any phage typing reaction with C1c.
"C1c + F116" denotes that IC was infected first with C1c and then, after separation of clones, with F116. The *in vitro* study—in contrast to the *in vivo* results—did not disclose any IC(C1c); consequently the clone used for F116 infection was as shown in the table corresponding to only C1c infection, i.e. sensitive to both F116 and C1c and with serogroup O 5 retained.

TABLE 2. *Changes in Phage Sensitivity of Individual Clones of* Pseudomonas aeruginosa *IC Strain and after an Interval*

IC phage pattern	Infecting phage	Time of examination relative to superinfection (days)	Number of colonies tested	Phage pattern of bacterial clones
M6, Me13 113 F116 C1c, C13† C21† H249 XVI	F116	2	46	
		7	47	M6 Me13 113 C1c, C13, H249 XVI 113 C1c, H249 M6, Me13 113 F116, C1c, C21 H249 XVI M6, Me13 113 F116, C1c, C13 H249 XVI M6 Me13 113 C1c, H249 M6, Me13 113 F116, C1c, C21 H249 P10 VII, XVI M6 Me13 113 C1c, H249 P10, VII XVI M6 Me13 113 C1c, C4 C13 H249 XVI
		20	18	M6 Me13 113 F116, C1c, H249 XVI M6 Me13 113 C1c, H249 XVI 113 C1c, H249
	C1c	1 or 2‡	35	
		18	104	M6 Me13 C13 XVI H249 M6, Me13 113 XVI M6, Me13 113 C13 C21 XVI F7 M6, M 13 113 C13 XVI M6 Me13 113 M6, Me13 M6, Me13 C13 Me13 113 C13 C21 XVI

† The reactions with the phages C13 and C21 were particularly variable in the original IC-strain *these phages have consequently been disregarded in the balance of gains and losses.*

‡ The port on of clones showing sensitiv ty or no reaction with the superinfecting phages was nearly the same on both days.

Exam nation of Rat Agglutinins

The presence of agglutinins against pseudomonas was examined in sera from all the rats. The sera were taken at least 3 weeks after termination of the experiments. Twofold saline dilutions from 1:5 were incubated over-night in Wassermann tubes at 37 C with an equal volume (0.5 ml) of antigens boiled for 2.5 hours. The sera were also examined by slide agglutination with 1:5 and 1:20 dilutions of the sera and live, over-night cultures. Reading was done after 2 minutes at room temperature. The antigens were the IC-strain, an IC (F116) (IC lysogenized by phage F116) IC (C1c) and the reference strains for the Habs serogroups O 5 O 6 and O 9

RESULTS

Changes in Lyso- and Serotype in vitro

Lysogenization of the IC-strain was attempted *in vitro* with the same phages as were used *in vivo* After C1c, there was no discernible serogroup or lysotype change in any of the colonies tested. After F116, there

*…solated from the Faeces of Mono-Contaminated Gnotobiotic Rats after Infection with the Phage F116 … with the Phage C1c**

No. of corresponding clones	Change in phage relative to IC — *Gains*	*Losses*	Sensitivity or resistance to phage superinfection‡
			^{S}F116 = 19 ^{R}F116 = 27 ^{S}C1c = 46
20		F116	^{S}F116 = 7
16		M6, M 13 F116, XVI	^{R}F116 = 40
3			^{S}C1c = 47
3			
2		F116 XVI	
1	P10, VII		
1	*P10 VII*	*F116*	
1	C4	F116	
10			^{S}F116 = 10
6		F116	^{R}*F116* = 8
2		M6, Me13 F116 XVI	^{R}C1c = 18
			^{S}F116 = 12 ^{R}F116 = 23 ^{S}C1c = 33
39		*113, F116, C1c, H249*	^{R}F116 = 104
30		F116, C1c, H249	^{R}C1c = 104
8		F116 C1c, H249	
2	F7	F116, C1c, H249	
2		F116, C1c, H249 XVI	
1		113, F116, C1c, H249 XVI	
1		113, F116, C1c, H249, XVI	
1		M6, F116, C1c, H249	

At various intervals after infection with the phages F116 and C1c, single bacterial clones were phage typed. In this table the emphasis is on the subsequent development of resistance to the *F116* or *C1c* and the concomitant changes in phage pattern. The table indicates results of Experiment I

‡ For instance, ^{S}F116 = 19 indicates that 19 clones were sensitive (S) to the superinfecting phage *F116*. ^{R}*F116* = 27 that no reaction (R = resistance) occurred with F116 in 27 clones.

was a change to serogroup O:6 in 6 of the 15 single clones examined (Table 1). These also changed lysotype; indeed, resistance to both F116 and C1c developed. This clone was lysogenic with a phage having a plaque morphology and host range resembling that of F116.

Since the IC (F116) (IC-strains lysogenized with phage F116) strains did not exhibit lysosensitivity to C1c, an O:3 clone was used for subsequent *in vitro* infection with C1c. This step mostly rendered clones which were indistinguishable from the progenitor but it is significant to note that three lysogenic isolates had serogroup O:9 and a markedly different lysotype. These 3 clones liberated phages which resembled C1c in plaque morphology; the host spectrum differed in two strong reactions, but three other reactions were as those seen in the case of C1c.

The clone used for infection with F116

TABLE 3 *Phage Sensitivity Pattern and Serogroup of Individual Clones of Pseudomonas aeruginosa IC Isolated from the Faeces of Gnotobiotic Rats after Superinfection with the Phage F116 and after an Interval also with the Phage C1c*

Experiment	Last phage added	Phage sensitivity pattern§	Sensitivity (S) or resistance (R) to superinfecting phages F116	C1c	Lysogenic	Serogroup	Number of corresponding clones
I	F116	M6, Me13 113 F116 C1c, C21 H249 PI0 VII XVI	S	S	ND‡	5	1
		M6 Me13 113 C1c, H249 P10 VII XVI	R	S	ND	5	6
		113 C1c, H249	R	S	ND	5	1
		M6, Me13 113 C1c, H249	R	S	ND	5	1
	C1c	M6 Me13 C13 XVI	R	R	ND	9	8
		M6 Me13 C13 XVI	R	R	ND	NT†	1
		M6 Me13 C13 XVI	R	R	ND	5	1
		M6, 113	R	R	ND	SA	1
		M6 Me13 113 H249 XVI	R	R	ND	5	1
II	F116	M6 Me13 C1c, XVI	R	S	+	5	10
		M6 Me13 113 C1c, H249 XVI	R	S	—	5	5
		M6 Me13 113 C1c, H249 XVI Z19	R	S	+	5	6
	C1c	113 C1c	R	S	+	5	1
		M6 M 13 113 C1c, H249 XVI	R	S	+	5	3
		NT	R	R	+	5	1
		M6 Me13 XVI	R	R	+	9	1
		M6 Me13 113 XVI	R	R	+	9	2
		M6	R	R	+	9	1
		NT	R	R	+	9	2

* Clones were selected on the basis of phage typing pattern such that all major patterns could be serogrouped and recovery of prophage attempted.

§ Phage sensitivity pattern should be compared with that of the original IC-strain M6, Me13, 113, F116 C1c, C13 C21 H249 XVI The reactions with C13 C21 were particularly variable in IC.

‡ ND = not examined.

† NT = non-typable.
SA = self agglutinable.

subsequent to C1c was indistinguishable from the original IC-strain, as evidenced by lysotyping serogrouping and non-lysogenicity After superinfection with F116 only lysogenic O-6 clones were found. The plaque morphology and lytic spectrum of the phages liberated from the clones were compatible with F116

Changes in Lyso- and Serotype in vivo

A In vivo *type stability before phage infection.* The pseudomonas colonization of the gut had been effective since the faeces contained 10^7–10^8 colony forming units per gram. The phage sensitivity pattern and serogroup of the IC-strain remained unchanged throughout a 10-week observation period in Experiment I and a one-week interval in Experiment II.

B Effect of infection with F116 In both Experiment I and Experiment II the serogroup remained unchanged throughout a three week observation period after F116 was given. Attempted induction of lysogenic phage failed (Table 2) Only minor changes in phage typing pattern were observed after introduction of F116 into the IC-monocon-

taminated animals. Two days after F116 in Experiment I 40 per cent of the isolates were still sensitive to F116 and 100 per cent to C1c. After 20 days, 10/18 strains had the phage sensitivity pattern and serogroup of the original IC-strain, the rest exhibited no lysis with F116. Losses or gains of lytic reactions were observed. Similar results were obtained also in the second *in vivo* experiment (Table 2-3)

C After subsequent superinfection with C1c Three weeks after the introduction of F116, no more changes appeared in the faecal bacteria. At the time of introducing the phage C1c, all isolates remained sensitive to it.

In both Experiment I and II clones with serogroup O:9 appeared (Table 3) All such clones examined were lysogenic. A number of lysogenic O:5 isolates were also found. The phages recovered from the O:9 strains were similar to C1c in plaque morphology but the host spectrum varied a little from that of the original C1c. Many of the O:5 clones rendered F116-like phages. Once in Experiment I a self-agglutinable strain with the phage type M6, 113 was isolated.

In both experiments, resistance to C1c developed quickly as determined in all isolates taken after only one day (Table 2) The changes in phage reactions in Table 2 have been recorded relative to the lysotype of the IC-strain, since more than one phage sensitivity pattern was present in the stools when C1c was introduced.

Examination of Lyophilized Strains

Six clones of serogroup O:9 from the *in vivo* Experiment II were lyophilized. When these were re-examined after storage for 1½ years at +4 C, all had reverted to serogroup O 5 and the IC-phage sensitivity pattern. Prophage could no more be induced.

Rat Agglutinins

Sera from the rats after completion of the experiments were free from agglutinins against pseudomonas as evidenced by both tube and slide techniques.

DISCUSSION

Changes in somatic antigens pursuant to lysogenization have been well documented in *Salmonella* (6 17 19) Using a number of combinations of phages and bacteria, *Holloway & Cooper* (9) *Phelps & Kurtz* (16) and *Liu* (13) have demonstrated seroconversion also in *P aeruginosa* after lysogenization *in vitro* Lysogenization has also resulted in pigment production, induction of proteolytic activity (13) and mucoid colonies (14)

The best explanation of the serogroup modification presently observed *in vivo* is phage conversion. Pseudolysogeny and carrier state may be considered, but these phenomena are unstable here several serial subcultivations of single colonies were performed before examinations for phage liberation, so that lysogenic conversion is believed to be more likely Attempts to induce prophage from the IC-strain before infection with F116 and C1c failed. All clones with serogroup specificities differing from the original O:5 of the IC-strain upon induction rendered phages resembling those used in the experiments. Phage were liberated from bacteria after both *in vitro* and *in vivo* infection with phages and resultant serogroup changes. In contrast to transduction, phage conversion fits well with the fact that the propagating strains for the phages F116 and C1c did not belong to the serogroup O:6 or O:9 observed after infection of the IC-strain. Phage conversion would also agree with the observation that the clones which *in vivo* had obtained O:9 after lyophilization and loss of prophage reverted to O:5

Loss of prophage upon lyophilization has also been observed by others. In view of the present findings that prophage may determine phage type and serogroup, *Beumer et al* (3) have actually indirectly demonstrated to what extent they may have consequences for pseudomonas. They showed that lyophilization resulted in phage type changes in 40 per cent of a series of *P aeruginosa* isolates. This clearly is a consequence of the high incidence of lysogeny (up to 90–100 per cent)

(7 10) in pseudomonas in which even poly lysogenization has been observed (18). Considering the rather disturbing frequency of changes in phage type, serogroup and pyocine type in serial isolates of pseudomonas from patients (3, 4) these findings would have considerable significance.

Although some changes induced *in vivo* were reproduced *in vitro* there were certain qualitative and quantitative differences between the two systems. Most notably O·6 clones were obtained by F116 infection *in vitro* but not *in vivo*. Although no attempts have been made to quantitate the frequency of the various types, it is still not likely that the marked differences have been obtained by pure chance. It could be that the O 6 trait here was an unstable character due to a high frequency of F116 loss. Another possibility would be that the O·6 clones, besides the new serogroup also acquired other characteristics of unknown nature which gave them an ecological handicap. Even without any such mechanism, however new strains would easily have difficulties in rising to a high proportion, since the faecal flora at the time of possible O·6 development already contained 10^7–10^8 bacterial colony forming units per gram faeces. Presence in relatively small numbers could escape detection in the present context. It seems relevant to note also that *Liu* (13) previously apparently has obtained different serogroups with the same combinations of pseudomonas and phage strains.

The question may also be raised why lysogeny was not detected after C1c was applied *in vitro* to IC. This is parallel to the findings of *Holloway et al.* (9) who also noted low rates of lysogenization with clear plaque mutants of pseudomonas phages.

It is a matter of speculation whether preceding lysogenization with F116 may increase the changes of phage conversion to O:9 by C1c. Since they were resistant to C1c, strains with F116 prophage were not used for the C1c superinfection *in vivo*. The IC-progeny used *in vitro* subsequent to F116 for C1c infection did not liberate phage and was consequently probably identical to the progenitor strain.

Robbins & *Uchida* (17) in *Salmonella* showed that successive lysogenization by two phages may render O-antigens which differ from those seen if each phage is used alone. Some temperate phages are only able to induce serogroup changes when another prophage is present. In *Salmonella Robbins* & *Uchida* showed specific impacts on cell wall composition. This was always expressed regardless, *i.e.* detectable by chemical procedures, but did not necessarily change the antigens. Such mechanisms may also be relevant for *P aeruginosa*.

An apparent change to a different serogroup and lysotype could also emerge from phage selection, *i.e.* survival of phage resistant mutants. In the present context this is unlikely. Before use, the IC-strain had been meticulously subcultivated to obtain a pure single-cell line. The complete stability in phage type and serogroup for as long as 10 weeks during monocontamination of the rats is also significant. It is unlikely that a selective pressure was exerted by the host *e.g.* by secretion of specific antibodies into the gastro-intestinal tract. A selective pressure whereby the host would excrete IgA antibodies in the gut is unlikely since production of IgA would most likely be parallel to IgM or IgG. The two latter components were probably absent since no agglutinating antibody was found in rat sera obtained *after* the termination of experiments.

Variations in typing results have been of considerable concern in *P aeruginosa* (2, 4) and have occasionally been so frequent that the usefulness of epidemiological typing methods could be questioned. It is of special relevance that apparent changes of serogroup strains of *P aeruginosa* has been observed previously (11, 12). The consequences of the present findings of serogroup variation and lysotype changes are many. Accordingly epidemiological types may change in patients even without superinfection with other nosocomial strains. Phage conversion or loss of a temperate phage with

pursuant serological change may both occur. Alterations in lysotype and serogroup from serial cultures of the same patient sites (3 4) may consequently be due to intrinsic changes of the flora and do not necessarily indicate either superinfection or a poor reproducibility of the typing method. The high frequency of lysogenic partly even polylysogenic, *P aeruginosa* strains will enhance the frequency of such capricious changes.

REFERENCES

1 *Bergan T* A new bacteriophage typing set for *Pseudomonas aeruginosa*. 1 Selection procedure Acta path. microbiol. scand. Sect. B, *80* 177–188, 1972.

2. *Bergan T & Lystad A* Reproducibility in bacteriophage sensitivity pattern of *Pseudomonas aeruginosa*. Acta path. microbiol. scand. Sect. B, *80* 345–350 1972.

3 *Bergan, T.* Epidemiological markers for *Pseudomonas aeruginosa*. 3 Comparison of bacteriophage typing, serogrouping, and pyocine typing on a heterogenous clinical material. Acta path. microbiol. scand. Sect. B, *81* 91–101 1973

4 *Bergan, T* Epidemiological typing methods of *Pseudomonas aeruginosa*. In: Brown, M. (Ed.) Resistance of *Pseudomonas aeruginosa*. John Wiley London In press 1975

5 *Brumer J Cotton E., Delmotte A., Millet M von Graevenitz W & Yourassowsky E* Ampleur des modifications du lysotype provoquées par la lyophilisation chez des souches de "*Pseudomonas aeruginosa*" Ann. Inst. Pasteur *122* 415–423 1972.

6 *Escobar M R. & Edwards P R* Lysogenic conversion of O antigens in *Salmonella* group C_1 Can. J Microbiol. *14* 453–458, 1968

7 *Feary T W Fisher E. & Fisher T N* Lysogeny and phage resistance in *Pseudomonas aeruginosa*. Proc. Soc. exp. biol. Med *113* 426–430 1963

8. *Habs I* Untersuchungen über die O-Antigene von *Pseudomonas aeruginosa*. Z. Hyg. Infekt Kr *144* 218–228, 1957

9 *Holloway B. W & Cooper G N.:* Lysogenic conversion in *Pseudomonas aeruginosa*. J Bact. *84* 1321–1324 1962.

10. *Holloway B. W., Egan J B. & Monk M* Lysogeny in *Pseudomonas aeruginosa*. Aust. J exp. Biol. *38*: 321–330 1960.

11 *Homma J Y., Shionoya H., Yamada, H Enomoto M & Miyao K.:* Changes in serotype of *Pseudomonas aeruginosa* Jap J exp. Med. *42* 171–172, 1972.

12. *Kawaharajo K.:* Changes in serotype of *Pseudomonas aeruginosa*. Jap. J exp. Med. *43* 25–276 1973

13 *Liu P V* Changes in somatic antigens of *Pseudomonas aeruginosa* induced by bacteriophages. J infect. Dis. *119* 237–246 1969

14 *Marti D R.* Mucoid variation in *Pseudomonas aeruginosa* induced by the action of phage. J Med. Microbiol. 6 111–118, 1973

15 *Midtvedt T & Trippestad A.* Opsonizing and bactericidal effects of sera from gnotobiotic and conventionalized rats on ³²P-labelled *E. coli* Acta path. microbiol. scand. Sect. B, *78* 1–5 1970.

16 *Phelps L. N & Kurtz H M* Lysogenic conversion of somatic antigens in *Pseudomonas aeruginosa*. Bact. Proc. 1964 p. 81

17 *Robbins P W & Uchida T* Studies on the chemical basis of the phage conversion of O-antigens in the E-group *Salmonellae* Biochem. *1* 323–335 1962.

18 *Shionoya H., Goto S Tsukamoto M & Homma J Y* Relationship between pyocine and temperate phage of *Pseudomonas aeruginosa*. I. Isolation of temperate phages from strain P 1 III and their characteristics. Jap. J exp. Med. *37*: 359–372, 1967

19 *Uetak H., Luria, S E. & B rrows J W.:* Conversion of somatic antigens in *Salmonella* by phage infection leading to lysis or lysogeny Virology *5* 68–91 1958.

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THE OCCURRENCE OF MYCOPLASMAS IN THE URINARY TRACT OF PATIENTS WITH CHRONIC PYELONEPHRITIS

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Thomsen, A. C. The occurrence of mycoplasmas in the urinary tract of patients with chronic pyelonephritis. Acta path. microbiol. scand. Sect. B 83 10–16, 1975.

Two groups of patients, each consisting of 40 were investigated for the occurrence of mycoplasmas in the urethra, bladder and upper urinary tract. Mycoplasmas were isolated significantly more frequently from the bladder urine of patients with chronic pyelonephritis than from patients with non-infectious urinary tract diseases. Furthermore mycoplasmas were isolated from the upper urinary tract of 5 patients with chronic pyelonephritis, while mycoplasmas could not be cultivated from the upper urinary tract of patients with non-infectious urinary tract diseases.

Key words Mycoplasmas; urinary tract chronic pyelonephritis.

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In 1937 *Dienes & Edsall* (4) isolated mycoplasmas from an abscess in the Bartholin gland. Since then, the frequent occurrence in the lower urinary tract of both large-colony mycoplasmas and T mycoplasmas (in the following referred to as *Ureaplasma urealyticum* (9)) and their possible aetiological implication in non-gonococcal urethritis has attracted a great deal of interest.

On the other hand, only a few investigators have reported on the occurrence of mycoplasmas in the bladder and the upper urinary tract.

Mårdh et al (6) isolated *Mycoplasma hominis* and *U urealyticum* from urine collected by suprapubic aspiration of the bladder urine from patients with chronic pyelonephritis. Using the same technique, *Wittleb et al.* (12) isolated mycoplasmas from urine specimens of patients with urinary tract infections. *Mycoplasma salivarium* has been isolated from the kidney of a patient with focal glomerulonephritis and focal pyelonephritis (7) and *M hominis* and *U urealyticum* from urine obtained through a ureteric catheter from a woman suffering from chronic pyelonephritis (12) None of these studies provided anything but suggestive evidence at best, for a pathogenic role of the mycoplasmas

A pathogenic effect of mycoplasmas in the upper urinary tract of rats has recently been demonstrated under experimental conditions. Acute pyelonephritis was produced by intracardial inoculation of *Mycoplasma arthriti-*

dis after ligature of the ureter (10). Moreover the *M. arthritidis* infection was shown to aggravate the effect of secondary infection with *Escherichia coli* (11).

The purpose of the present investigation was to determine the occurrence of mycoplasmas in the urinary tract of patients with chronic pyelonephritis as compared to patients with non-infectious urinary tract disease.

MATERIAL AND METHODS

Group I. Patients with Non-Infectious Urinary Tract Disease

Thirty women and 10 men were examined. 23 patients had concrements in the upper urinary tract, 5 had tumours, 7 malformations, and 5 suffered from diseases of the bladder (tumours, neurogenic bladder-diseases). None of the patients revealed any signs of infection of the urinary tract such as elevated temperature or ESR, abnormal urine sediment, lumbar pain, dysuria or pollakisuria.

The youngest patient was 19 and the oldest 78 years of age. Two of them had been treated with antibiotics (penicillin and nitrofurantoin) during the last month before collection of specimens, but none of the antibiotics used were mycoplasma inhibitors.

The patients of this group were selected, to make them comparable with respect to age and sex to those of group II who were unselected. All selections were made before the results of cultivation were known.

Group II. Patients with Chronic Pyelonephritis

Thirty women and 10 men were examined. The diagnosis of chronic pyelonephritis was based on the occurrence of at least three of the following six criteria: 1) An anamnestic story of cystitis and attacks of acute pyelonephritis; 2) a urine sediment containing leucocytes; 3) significant bacteriuria; 4) a biopsy showing histological lesions of chronic pyelonephritis; 5) impaired function of the kidneys, and 6) the demonstration by X-ray examination of irregular shadow of the kidney and clubbing of the calyces.

The age of the patients ranged from 22 to 85 years with an equal distribution throughout the group. Fourteen patients had been treated with antibiotics within the last month, although not with compounds known to possess mycoplasma inhibiting effects.

Sampling

Group I. Specimens were taken from the urethra, bladder and the upper urinary tract.

Specimens from the urethra were taken by rotating sterile cotton-tipped swabs in the external orifieum.

For sampling of urine, the patients were catheterized and a mid portion of the urine (bladder urine) collected for cultivation.

From the upper urinary tract, specimens were taken during operation: cotton-tipped swabs were rotated in the pelvis renis, or urine was collected from the pelvis through aspiration or through a catheter.

Group II. According to the method of sampling this group was divided into three subgroups.

) From 14 patients, specimens from the urethra, bladder and upper urinary tract were collected during operation as described for group I.

From the remaining 26 patients, bladder urine was at first taken by catheterization and cultivated for mycoplasmas.

b) Seven of these 26 patients had mycoplasmas in the bladder urine, and the examination was therefore extended to include specimens from the upper urinary tract and the urethra. Urine from the upper urinary tract was collected through bilateral ureteric catheterization, and the urine was divided into an initial-, a middle- and a terminal portion. Specimens from the urethra were taken as described for group I.

c) Nineteen patients did apparently not harbour mycoplasmas in the bladder urine and no further examination was done in these cases.

The pH of all urine specimens was determined.

All samples were inoculated into growth media within one hour.

Cultivation and Identification

Large-colony Mycoplasmas

The growth medium (B) used in the present study has been described earlier (5). Solid medium was prepared by adding Ionagar No. 2 (Oxoid) 1.2 per cent, and semisolid medium by adding 0.1 per cent of Ionagar.

Swabs were streaked onto solid medium and then deposited in semisolid medium.

Cultures in semisolid medium were incubated in atmospheric air at 37° C and plating was performed after 3 days incubation. Dublicate sets of plates were incubated at 37° C in candle jars and in an atmosphere of 95 per cent N_2 plus 5 per cent CO_2. The plates were incubated for 4 days before examination for growth under a stereomicroscope. If no colonies were found, the plates were incubated for a further 4 days before repeated examination.

Following a preliminary grouping according to biochemical properties (fermentation of glucose,

TABLE 1 *The Occurrence of Mycoplasmas in 40 Patients with non Infectious Urinary Tract Diseases and in 40 Patients with Chronic Pyelonephritis*

Localization			Urethra*			Bladder			Upper urinary tract*	
Mycoplasma		species	*M hominis*	*M fermen-tans*	*U urealy-ticum*	*M hominis*	*M fermentans*	*U urealy-ticum*	*M hominis ticum*	*U urealy-*
Group I (Patients with non infectious urinary tract diseases)		10 ♂	0	0	3	0	0	0	0	0
		30 ♀	1	0	5	0	0	2	0	0
Total		40	1	0	8	0	0	2	0	0
Group II (Patients with chronic pyelonephritis)	a	3 ♂	0	0	0	0	0	0	0	0
		11 ♀	3‡	1	8‡	3‡	1	8‡	1	2
	b	1 ♂	0	0	1	0	0	1	0	0
		6 ♀	4	0	2	4	0	2	2	0
	c	6 ♂	Not investigated			0	0	0	Not investigated	
		13 ♀				0	0	0		
Total positive for mycoplasmas/total investigated			7/21‡	1/21	11/21‡	7/40‡	1/40	11/40‡	3/21	2/21

* Indications of the occurrence of a mycoplasmal species in more than one anatomical location refer throughout to the same patients.
‡ A mixed flora of *M hominis* and *U urealyticum* was found in three of these patients.

hydrolysis of arginine, reduction of tetrazolium and production of phosphatase) the mycoplasma isolates were identified by the indirect epi-immunofluorescence (6) and growth inhibition tests (3)

U urealyticum

The medium of Shepard as modified by *Black* (2) medium (8) was used. For solid medium 1.2 per cent Ionagar No. 2 (Oxoid) was added.

The specimens were inoculated both into broth and onto solid medium. The broth was incubated at 37 C for 24 hours after which subcultivation onto plates was performed. A duplicate set of plates were incubated at 37 C in 90 per cent atmospheric air plus 10 per cent CO and in an atmosphere of 95 per cent N_2 plus 5 per cent CO_2, respectively

Examination for growth was done on the third and the fifth day using a stereomicroscope.

The isolates were tested for hydrolysis of urea, and isolates from patients harbouring *U urealyticum* in the upper urinary tract were typed serologically by indirect epi-immunofluorescence (1) and by growth inhibition (2)

Quantitative Estimates

The number of colonies appearing after streaking of the swabs on the agar plates were recorded as + + + for >100 + + for 10–100 and + for 1–10 colonies.

The number of colony forming units/ml (c.f.u./ml) of mycoplasmas in urine specimens were determined using a serial ten-fold dilution of the urine followed by plating with a calibrated loop (0 01 ml)

Bacteria

All specimens were cultivated for bacteria using blood agar lactose bromthymol-blue aga and chocolat agar plates. The plates were incubated t 37 C aerobically and anaerobically

RESULTS

Cultivation for Mycoplasmas

Group I Patients with Non-infectious Diseases of the Urinary Tract

Mycoplasmas were found in the urethra of 9 and in the bladder urine of two out of the 40 patients examined. Mycoplasmas were not recovered in any case from the upper urinary tract in this group of patients (Table 1)

Group II Patients with Chronic Pyelonephritis

Subgroup a (specimens collected during operation) This subgroup included 3 male and 11 female patients. Mycoplasmas were not recovered from the male patients. From 9 female patients, mycoplasmas were cultivated from the urethra as well as from the bladder urine. From the upper urinary tract, mycoplasmas were isolated from three patients (Table 1)

Subgroup b (specimens collected by ureteric catheterization) This subgroup included 1 male and 6 female patients. Mycoplasmas were isolated from the urethra and the bladder urine of the male patient.

The six female patients harboured mycoplasmas in the urethra and the bladder urine. Two of these patients also harboured mycoplasmas in the upper urinary tract (Table 1)

Subgroup c (only bladder urine was investigated) Mycoplasmas were not isolated from any of the 6 male and 13 female patients in this subgroup (Table 1)

Altogether mycoplasmas were recovered from the urethra of 16 out of 21 from the bladder urine of 16 out of 40 and from the upper urinary tract of 5 out of 21 patients examined.

Mycoplasmas were the only isolates from the upper urinary tract of four of the patients and two of these, both harbouring *M hominis* revealed signs of an acute exacerbation (Table 2 and 3)

No relationship between the pH of the urine and the occurrence of mycoplasmas was found in any group. Neither was the occurrence of mycoplasmas correlated to a certain age group

The occurrence of mycoplasmas did not seem to have special affinity to the occurrence of a certain species of bacteria or to a bacteria free urinary tract.

Two patients from whom both *M hominis* and *U urealyticum* were isolated, had been treated with nitrofurantoin and ampicillin.

TABLE 2. *Results of Cultivation for Mycoplasmas and Bacteri from 5 Female Patients Harbouring Mycoplasmas in the Upper Urinary Tract*

Case No.	Cultivation results: Urethra	No. of colonies	Bladder	No. of colonies	Upper urinary tract	No. of colonies	Method used for sampling of specimens from the upper urinary tract
1	*U u ealyticum* type I	+	*U urealyticum* type I	3×10^{4}	*U urealyticum* type I	+ +	Swabs from the pelvis renis
	P vulgaris		*P vulgaris*	$<10^{5}$/ml			
2	*U realyticum* type III	+	*U urealyticum* type III	2×10^{5}	*U urealyticum* type III	+ +	Swabs from the pelvis renis
	S albus		*S albus*	$>10^{5}$/ml	*S a bus*		
3	*M hominis*	+ + +	*M hominis*	4×10^{5}	*M hominis*	2×10^{5}	Urine aspirated from the pelvis renis
4	*M hominis*	+ +	*M hominis*	5×10^{3}			*Ureteric catheterization*
	U urealyticum	+ +			*M hominis*		Right side Initial portion
	S albus				0		Middle portion
					0		Terminal portion
					M hominis	2×10^{3}	Left side Initial portion
					M hominis	10^{3}	Middle portion
					M hominis	10^{3}	Terminal portion
5	*M hominis*	+ +	*M hominis*	5×10^{3}			*Ureteri catheterization*
	E. coli		*E coli*	$<10^{5}$/ml	*M hominis*	3×10	Right side Initial portion
	S albus				*E. coli*		
					M hominis	10^{3}	Middle portion
					M hominis	3×10^{3}	Terminal portion
					M hominis	2×10^{3}	Left side Initial portion
					E. coli		
					0		Middle portion
					0		Terminal portion

Swabs recorded as + + + for >100 + + for 10–100, and + for 1–10 colonies.
U rine pecimens recorded as the number of f u./ml.

TABLE 5. *Clinical and Laboratory Information of 5 Female Patients Harbouring Mycoplasmas in the Upper Urinary Tract*

Case No.	Age	Duration of chronic pyelonephritis in years	Signs of acute exacerbation	Fever	Pyuria	Antibodies against mycoplasmas isolated from the upper urinary tract*		
						Days‖	Method†	Titer
	50	15	—	—	—	0 66	MIT	0 0
	58	1/2	—	—	—	0 166	MIT	32 32
	28	10	+	+	+	0 4 127	IHA	32 128 16
4	43	7	+	+	—	0 20 121	IHA	256 512 16
5	59	17	+	+	+	0 6 93	IHA	32 512 128

* Further details will be described in following paper

‖ Days between collection of specimens for cultivation and serum for determination of antibodies.

† MIT = Metabolic inhibition test. IHA = Indirect haemagglutination test.

‡ The patient had received antibiotics, i.e. nitrofurantoin.

Cultivation for Bacteria

Group I

Neither significant bacteriuria ($>10^5$/ml) nor the occurrence of bacteria in the upper urinary tract could be demonstrated in any of the 40 patients examined.

Group II

Nineteen of the 40 patients examined had significant bacteriuria. The bacteria were identified as *E. coli* in 12 and as *Proteus vulgaris* in 7 of these cases. Four patients had *E. coli* and two *P. vulgaris* in the upper urinary tract as well.

DISCUSSION

The patients included in the group with non-infectious urinary tract disease were selected so that they were comparable in respect to age and sex with the patients in the group with chronic pyelonephritis. This seemed reasonable as it is well-known that the occurrence of mycoplasmas in the urogenital tract is correlated to age and sex.

Mycoplasmas were isolated from 16 out of 40 specimens of the bladder urine (40 per cent) from patients with chronic pyelonephritis. From the bladder urine of 40 patients with non-infectious urinary tract disease, mycoplasmas were isolated only twice (5 per cent). This difference between the two groups of patients is significant ($\chi^2 = 14.05$, $p<0.01$).

Five out of 40 patients with chronic pyelonephritis harboured mycoplasmas in the upper urinary tract (13 per cent). The mycoplasmas were isolated in pure culture in four cases, but one of the patients had received antibiotics and thus, bacteria might have been present before collection of specimens. Mycoplasmas were not isolated from the upper urinary tract of any of the patients with non-infectious urinary tract disease.

In three cases, the specimens from the up-

per urinary tract containing mycoplasmas were collected during surgery and the possibility of contamination from the lower urinary tract can thus be excluded in these cases. In the remaining two cases, a contamination from the bladder to the upper urinary tract through the ureteric catheter is *unlikely since only a slight decrease in the* number of c.f.u./ml of mycoplasmas was found to occur in the interval between the drawing of the initial and the terminal portion of urine through the ureteric catheter on the side positive for mycoplasmas.

Moreover in samples from the opposite ureter mycoplasmas and bacteria could be isolated from the initial portion of urine only

The number of c.f.u./ml of mycoplasmas in the bladder urine did not indicate anything about the presence of mycoplasmas in the upper urinary tract. Several specimens of bladder urine from patients with no mycoplasmas in the upper urinary tract contained a higher number of c.f.u./ml of mycoplasmas than specimens of bladder urine from patients harbouring mycoplasmas in the upper urinary tract.

It is noteworthy that all patients harbouring *M. hominis* in the upper urinary tract showed signs of an acute exacerbation of pyelonephritis. This is suggestive of a connection between the occurrence of *M. hominis* and physical signs of pyelonephritis, and it is supported by the demonstration in two of these patients of a four fold change in the antibody titre against *M. hominis* (Table 3)

However despite the isolation of mycoplasmas from the upper urinary tract of some patients with chronic pyelonephritis, as opposed to the negative findings in patients with non-infectious disease, it is not possible to conclude from the present study whether the mycoplasmas are involved in the production of the pathological lesions or whether these merely enhance the invasion of mycoplasmas.

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REFERENCES

1 *Black F T.*, Serological methods for classification of human T-mycoplasmas. Fifth Internat. Congress Inf. Dis. *1* 407–411 1970.

2 *Black F T* Modifications of the growth inhibition test and its application to human T mycoplasmas. Appl. Microbiol *25* 528–533 1973.

3 *Clyde W D., Jr.*, Mycoplasma species identification based upon growth inhibition by specific antisera. J Immunol. *92* 958–965, 1964

4 *Dienes L. & Edsall, G* Observations on L organisms of Klieneberger Proc. soc. exp. biol. med. *36* 740–745 1937

5 *Erno H & Stipkovits L.* Bovine mycoplasmas Cultural and biochemical studies. II. Acta vet. scand. *14* 450–463 1973

6. *Mårdh P.-A., Lohi, A. & Fritz H* Mycoplasma in urine collected by suprapubic aspiration. Acta Med. Scand. *191* 91–95 1972.

7 *Paskas W N* The role of mycoplasma in *some unusual conditions of the kidney and the* urinary tract. Ann. N Y Acad. Sci. *170* 786–793 1970.

8. *Rosendal, S & Black F T* Direct and indirect immunofluorescence of unfixed and fixed mycoplasma colonies. Acta path. microbiol. scand Sect. B, *80* 615–622 1972.

9 *Shepard M C Lunceford C D., Ford D K. Purcell R H Taylor Robinson D Razin S & Black F T Ureaplasma urealyticum* (Gen. nov sp. nov) Proposed nomenclature for the human T (T-strain) myco*plasmas Int J Syst Bacteriol 24* 160–171 1974

10 *Thomsen A. C Rosendal S & Thomsen O F* Mycoplasmosis Experimental pyelonephritis in rats. Acta path microbiol. scand. Sect. A, *81* 379–380, 1973

11 *Thomsen, A C & Rosendal S* Mycoplasmosis Experimental pyelonephritis in rats. The effect of secondary infection with *Escherichia coli*. Acta path. microbiol. scand. Sect. B, *82* 94–98 1974

12 *Wittlob W Färber I Thieler H & Bermöhr Th* Nachweis von Mykoplasmen in höheren Abschnitten der ableitenden Harnwege. Zbl Bakt., I Abt. Orig *208* 427–430, 1968.

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QUANTITATIVE STUDIES OF DIRECT AND INDIRECT IMMUNOFLUORESCENT STAINING OF SALMONELLA BACTERIA

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Karlsson, K.-A., Thore, A. & Kudynowski, J. Quantitative studies of direct and indirect immunofluorescent staining of Salmonella bacteria. Acta path. microbiol. scand. Sect. B, 83 17–24 1975

Salmonella bacteria were stained with serial dilutions of anti-Salmonella conjugates of different F/P ratios and the staining intensity was measured quantitatively in an ultramicrofluorometer. In any given dilution of the conjugates, a stronger fluorescence was obtained with the more highly labelled conjugates. The dependence of fluorescence intensity on F/P ratio varied with the dilution of the conjugate. Similar results were obtained by the indirect immunofluorescence method. In four bacterial systems the direct and indirect immunofluorescent staining methods were compared quantitatively. The indirect method was 5 to 30 times more sensitive than the direct comparing the last dilutions giving a positive reaction by visual observations. The standard deviation of the intensity values of the stained bacterial cells was between 10 and 40 per cent of the mean. Different sources of variation in the quantitative measurement technique are discussed.

Key words: Salmonella; quantitative immunofluorescent staining.

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Among microorganisms, quantitative immunofluorescence has predominantly been used to study the antigenic relationships of certain parasites (8). In virology quantitative immunofluorescence has been shown to be of value in evaluating different staining procedures (7) and in a quantitative way state the amount of antigen in virus infected cells (23). Recently quantitative immunofluorescence of bacterial antigens has been reported in connection with the development of new measuring techniques (13, 15, 21, 22).

In bacteriological applications of the immunofluorescence technique the direct method has been the one most often used. It might however in some situations be advantageous to use the more sensitive indirect method. From practical points of view it is also an advantage to prepare and store one single labelled reagent which can be used in conjunction with unlabelled antisera of different kinds. In bacterial systems, quantitative comparisons of these staining procedures have not been described, but such measurements might be expected to afford better criteria for a choice between the methods.

The object of the present study was to evaluate in a quantitative way the effect of different F/P ratios on the brightness of the specific staining reactions of Salmonella bacteria and to compare quantitatively the immunofluorescence obtained by the direct and indirect method for some salmonella strains.

MATERIAL AND METHODS

Strains. S typhimurium S cubana S aberdeen S dublin and one strain (My 79) of *Yersinia enterocolitica* belonging to O group V (16)

Antigens. For the production of antisera, broth cultures incubated at 37 C for 18–20 hours and treated with 0.5 per cent formalin were used. The antigens for O and H agglutination were prepared according to *Kauffmann* (14)

Antisera were produced in rabbits according to methods described earlier (12)

FITC-conjugated antirabbit globulin (sheep) from the National Bacteriological Laboratory Stockholm, was used. The reactivity of the conjugate was determined by chessboard titration (4)

Non specific staining (NSS) of the conjugate was checked with normal rabbit serum and saline. Only in the dilutions 1:2 and 1 4 of the conjugate a weak (+) NSS was established. The plateau end point titre of the conjugate was 1 64 using the four antisera concerned. Thus an ppropriate working-dilution was determined to 1 8.

Preparation of conjugates. The globulin fractions of the antisera were obtained by two precipitations in $(NH_4)_2SO_4$ at a final concentration of 40 per cent saturation. The ammonium sulphate was removed from the redissolved globulin by dialysis against phosphate-buffered saline (pH 7 4) The protein concentration of the globulin was determined by the ultraviolet absorption method (11) The globulin fractions were labelled with fluorescein isothiocyanate isomer I in pure crystalline form (Baltimore Biological Laboratory) The conjugation was carried out essentially according to the technique described by *Nairn* (19) However the amount of FITC was increased to 0 1 mg per mg protein and was allowed to react with the protein in the cold (0–4 C) for 2 hours.

Unreacted FITC was removed by passage through a Sephadex G-25 (coarse) column and remaining precipitate in the conjugate was removed by centrifugation. This procedure resulted in preparations w th F/P ratios of 11–14 μg FITC/mg protein as determined according to *Wells et al.* (24) Aggl tination titres of the conjugates were determined in tubes as described by *Kauffmann* (14) and the results are shown in Table 1

If F/P alues other han this were desired it was achieved by varying the amount of FITC added during labelling and by varying the time of conjugation.

Losses of agglutination titre did not in any case result from the labelling. The anti-globulin conjugate used had an F/P ratio of 6.5 (μg/mg *Wells et al.*) In one experiment the anti-globulin conjugate was further conjugated with FITC to obtain a higher F/P ratio.

Determination of F/P ratios. Since an accurate determination of F/P values was essential in experiments where the influence of various degrees of labelling was studied, F/P values were also determined using fluorescein diacetate dissolved in 0 1 M NaOH as reference (17) The amount of protein was determined by means of the biuret reaction with optical readings at 560 nm (9) The F/P ratios were calculated from the FITC and protein measurements and expressed as μg FITC/mg protein.

In Table 2 a comparison between the two methods for F/P determination is presented. The values obtained according to *McKinney et al.* (17) and the biuret reaction are used in Fig. 2, whereas in the other figures and tables only F/P values according to *Wells et al* (24) are used.

Stability of conjugates. The measurements of immunofluorescence of antibacterial conjugates kept in solution at +4 C in the presence of merthiolate (1 10 000) during one month were in agreement with the results obtained with freshly prepared conjugates. On the other hand there was a considerable reduction of the intensity of fluorescence after storage for 3 months (13)

Immunofluorescent staining Bacterial cells grown in broth were collected by centrifugation, washed once and re-suspended in saline Smears prepared from diluted antigen suspensions were fixed in a mixture of alcohol acetone and formalin (60:30 10) for 2 minutes and then immersed briefly in 95 per cent ethanol. Twofold dilutions of conjugate or serum were prepared in saline and a drop of the reagent concerned was spread over each antigen on the slides. The slides were incubated for 30 minutes in a moist chamber at 37 C and then washed twice in PBS pH 7.6, remaining 15 minutes in each bath. In the indirect test, the FITC-conjugated anti-rabbit globulin was applied for 30 minutes at 37 C. The slides were washed as described above and stained preparations were mounted with 50 per cent glycerol containing 25 mM KH_2PO_4, 25 mM tris, 100 mM NaCl, pH 8.2.

Microscopy Slides were read in a Zeiss fluorescence microscope with incident light using an HBO 200 lamp as light source. Exciter filter KP 500 and barrier filter No 50 were used The visual observations of the staining reactions were graded in the conventional way between 4+ and 1+ The 4+ and 3+ reactions are considered

TABLE 1 *Characteristics of Antibacterial Conjugates*

Antibacterial conjugates	Agglutination titres O	Agglutination titres H	F/P ratio* (µg FITC/mg protein)
S typhi murium	640	6400	13
S cubana	640	12800	12
S aberdeen	320	6400	11.5
Y enterocolitica	640	12800	13

* F/P determination according to *Wells et al.* (24)

TABLE 2 *F/P Determinations of Antibacterial Conjugates with Different F/P Ratios*

FITC-conjugated anti *S typhi murium* serum	Agglutination titres		F/P ratio (µg FITC/mg protein)	
	O	H	Method I*	Method II§
I	160	2560	4.5	4.5
II	160	2560	11.2	15 1
III	160	2560	15 7	19.5
IV	160	2560	20.0	23 4

F/P determination according to *Wells et al.* (24)
§ FITC determination according to *M Kinney t al.* (17)
Protein determination by the biuret method (9)

as positive whereas 2+ and 1+ are considered negative.

Photometry The measurement of immunofluorescence in individual bacteria offers technical dif ficulties because of the small size of the objects and because of the rather rapid quenching of the fluorescence by the UV irradiation.

The previously described ultramicrofluorometer (5, 13) was well suited for this type of work. In this instrument the object is UV-illuminated only during the very short time of photometric measurement which is made with a sensitive photometer arrangement. The light registering unit was a Zeiss Microscope Photometer MPM, with the photometer head replaced by the optical arrangement described by *Caspersson et al.* (5) By the aid of a special field limiting system the area to be measured—as a rule an individual bacterium—is optically isolated in a rapid and convenient way The measuring field is adjustable to different forms and sizes of the bacteria. Thus, during the measurement of one bacterium no others are illuminated.

The basic fluorescence microscope equipment used for ultramicrofluorometric measurements was from Zeiss, Oberkochen with incident illumination using a mercury lamp (HBO 100) as light source Exciter filters 2.5 mm BG 3 + ..5 mm KG 1 and barrier filter No 50 were used. The epi-illuminator had the dichroic mirror Fl 500. The standardization of the instrument was performed with the aid of fluorescent uranyl glass (GG 17)

The measurement of the immunofluorescent preparations were performed as follows. The bacterium to be measured was localized during phase illumination and the measuring field adjusted to the bacterium. Then the exciting UV light was allowed to illuminate the preparation and the fluorescence intensity was registered immediately thus minimizing any quenching effect. To obtain values for background fluorescence the area adjacent to the bacterium was measured in the same way and all fluorescence intensities were corrected by this value.

Measurements throughout this study were always carried out on 10 separate bacteria in each preparation. It was therefore possible to evaluate the measurements statistically In Fig. 1 the standard deviations of measurements on several preparations are plotted as percent of the total fluorescence intensity The linear regression lines plotted according to least square method coincide within the limits of error regardless of the various experimental conditions used in the study It is evident that the relative deviations become smaller with higher fluorescence intensities which would indicate that reproducible measurements are most easily obtained in this range. Standard deviations

2*

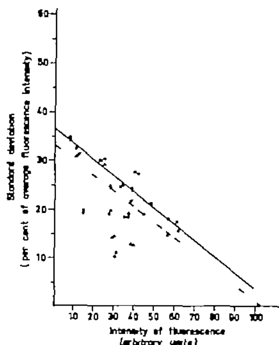


Fig 1 Statistical evaluation of microfluorometric measurements. Each of the points in the diagram represent the relative standard deviation calculated from measurements on bacteria in each preparation. Altogether measurements on 109 preparations made by the direct (▲) and indirect (●) immunofluorescent methods are included. The lines represent linear regression lines calculated for the direct (broken line) and indirect (solid line) method.

usually vary between 10 and 40 per cent of the average whereas the standard error of the mean is about 3–12 per cent. This means that single measurements of bacterial cells will not afford reliable results whereas the averages determined from measurements of 10 cells are reasonably accurate.

RESULTS

In Table 3 average fluorescence intensities from 10 series of experiments with direct and 5 series with indirect immunofluorescence of various Salmonella strains were recorded and classified according to visual observations. The table demonstrates that the results obtained by conventional visual observation may equally well be expressed as well-defined intervals of fluorescence intensity regardless of staining method.

Fig. 2 depicts results from measurements

TABLE 3. *Comparison of Visual Observations and Microfluorometry of Immunofluorescent Salmonella Bacteria*

Average intensity ± SD (arbitrary units) (numbers in brackets indicate numbers of slides examined)		Visual observation
Direct method§	Indirect method†	
37 ± 14 (31)	46 ± 20 (43)	+ + + +
24 ± 6 (11)	28 ± 9 (8)	+ + +
14 ± 4 (18)	14 ± 7 (9)	+ +
7 ± 4 (22)	8 ± 8 (7)	+

* 10 individual bacteria were measured in each slide.

§ Measurements were performed in titrations of the following bacterial systems *S aberdeen S cubana S typhimurium S dublin* and *S muenchen*. Range of F/P values was 6.5–13

† Measurements were performed in titrations of the following bacterial systems *S aberdeen S cubana S typhimurium*, and *Yersinia enterocolitica*. The F/P value of the anti γ globulin conjugate was 6.5

of cells of *S typhimurium* stained with dilutions of a homologous conjugate labelled with various amounts of fluorescein isothiocyanate. It is evident that in any given dilution of the conjugates the stronger fluorescence is obtained with the more highly labelled conjugates. However the dependence of fluorescence intensity on F/P ratio varies with the dilution of the conjugate. Thus, at a dilution of 1:40 the conjugate of F/P 23.4 results in a ten fold higher fluorescence intensity than the conjugate of F/P 4.3 whereas at the dilution 1:80 the difference is only five fold.

In Fig 3 results from an experiment with conjugates of various *F/P* using the *indirect* method are shown. Also with the indirect IF method the brightness of the stained bacterial cells is dependent on the *F/P* ratio of the conjugate used. As comparison, a series of measurments were made on the same bacteria stained by the direct method (lower curve) It may be seen that the fluorescence intensity is much lower if the direct method is used as compared to the equally labelled conjugate used in the indirect method. To compare the performance of direct and indirect IF titrations of three Salmon-

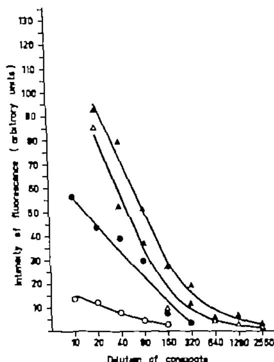


Fig 2 The influence of F/P ratio on the direct staining of *S typhi murium*. The following antibacterial conjugates were used (○——○) F/P 4.3 (●——●) F/P 15 1; (△——△) F/P 19.3 (▲——▲) F/P 23 4 and the fluorescence intensity obtained when staining with various dilutions of these conjugates was recorded.

ella strains were made. The results are shown in Fig 4 With the intermediate F/P ratios used in this experiment the indirect method in all cases resulted in higher fluorescence intensities at any given dilution. With *S cubana* (4a) and *S typhi murium* (4b) there was a marked tendency towards plateau shaped curves at higher concentrations of reagents. This was observed whether the direct or the indirect methods were used. With *S aberdeen* (4c) however a plateau was not observed, possibly due to the relatively low titre of the reagents for this organism. With *S typhi murium* (4b) a distinct prozone phenomenon was observed whether direct or indirect methods were used.

At dilutions higher than those resulting in prozone or plateau phenomena, the curves of the indirect and direct staining run approximately parallel with a slope of about 10 intensity units per twofold dilution of the reagents. Due to the plateau shape of the curves, the distance between the direct and indirect curves for *S cubana* and *S typhi murium* is decreased at higher concentrations of the reagents, making the staining by the two methods comparable in intensity

From a practical point of view the most interesting differences in fluorescence intensity between direct and indirect methods are found in the region of 25 intensity units. As shown in Table 3 this intensity corresponds to a visual observation of a 3+ staining reaction. In titration, this is the commonly used definition of the immunofluorescence end point titre. At this value, the distance of the curves for direct and indirect IF corresponds to dilution factors of 4 16 and 32 in *S aberdeen S typhi murium* and *S cubana* respectively This is reflected in the working dilutions chosen in practice.

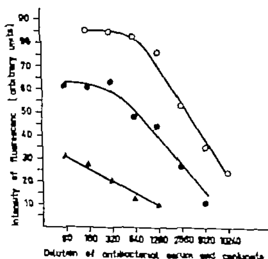


Fig 3 The influence of F/P ratio on the indirect staining of *S typhi murium* The following anti rabbit conjugates were used: (●——●) F/P 6.5; (○——○) F/P 14 at a dilution of 1:8. The dilution of the unlabelled serum was then varied and the fluorescence intensity was recorded.
By way of comparison an experiment with the same strain was performed using the direct method (▲——▲) The F/P ratio of the antibacterial conjugate was 13.

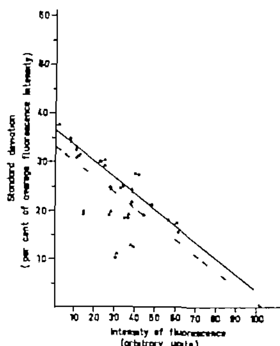


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zone phenomena and plateau formation could be observed, using both indirect and direct immunofluorescence. Similar observations in other bacterial and viral systems have been reported (1 7). *Cherry* (6) has proposed that the levelling off in fluorescence intensity at lower dilutions of immunofluorescent reagents might be due to a quenching effect on the fluorescence. Quantitative microfluorometry might provide useful information pertinent to this discussion.

An important parameter by which to evaluate conjugates is the F/P ratio. It seems obvious that a higher degree of labelling of the conjugate i.e. a high F/P ratio results in brighter fluorescence of the stained preparation. On the other hand, in many applications too high F/P ratios may lead to unwanted non-specific staining. In bacteriological applications non-specific staining due to high F/P ratios as a rule does not present great problems and therefore usually a wide choice of F/P ratios is afforded. There is an increasing interest in using polyvalent conjugates directed towards several Salmonella antigens to detect Salmonella in foods by IF technique. To cover the multiplicity of antigens within the Salmonella group it may be necessary in order to maintain immunofluorescent titres to use conjugates with F/P ratios resulting in maximal specific fluorescence. The results presented in this report show that stronger fluorescence is obtained with the more labelled conjugates at any given dilution at least up to a F/P ratio of about 25. This is partly reflected in higher end point titres of the conjugates. Similar studies of an *E. coli* system have shown that the end point titre of specific staining had reached a maximum already at an F/P ratio of approximately 6 whereas Streptococcus conjugates seemed to have their maximum at an F/P ratio of 28 to 33 (20).

In indirect immunofluorescent staining with human antibody systems it has been verified that the titre of primary antibodies varies with the F/P ratio of the conjugates (2, 3, 4, 10). Although the results in this report are limited they clearly indicate that the titres of primary antibodies obtained in indirect IF staining of Salmonella bacteria also varies with the F/P ratio of the conjugates.

It is a well-known fact that the indirect immunofluorescence method is substantially more sensitive than the direct method (8, 19) and this difference has been demonstrated quantitatively in various viral systems (7). The increased sensitivity of the indirect method is confirmed in the Salmonella systems investigated in this study. However great differences in this respect exist between the different bacterial systems. It must also be noted that the differences in sensitivity as demonstrated in this investigation must be judged by the degree of labelling of the conjugates used. When the degree of labelling is taken into account the greater sensitivity of the indirect method is even more pronounced.

In quantitative measurements of immunofluorescence many sources of variation exist. These variations may be due to biological, technical or instrumental factors such as inhomogeneity of the smears, imperfect randomization, fading of fluorescence. The relative sizes of these different sources of variation have not been determined, but it is very likely that inhomogeneity of the bacterial antigens plays a dominant role. By visual observation it may be noticed that bacterial cells in a stained smear may vary in size as well as in intensity of fluorescence. Nevertheless, the present study has demonstrated that, provided the number of cells measured is sufficient, useful information may be obtained by quantitative microfluorometry.

The introduction of quantitative methods in bacterial immunofluorescence may in the future provide more exact information of the underlying serological reactions. Such information is important also from practical points of view in the search for improved reagents and staining procedures. It might also prove an important tool in the standardization of procedures and reagents from different laboratories by facilitating comparisons of results.

We gratefully acknowledge the generosity of professor *Torbjörn Caspersson* in placing at our disposal the ultramicrofluorimetric instrumentation at The Institute for Medical Cell Research and Genetics, and for valuable help and discussions throughout this work.

REFERENCES

1 *Barnett E. V* Staining activity of conjugates in relation to their antibody content. In Holborow E. J Standardization in Immunofluorescence. Blackwell Scientific Publ. Oxford, England 1970, pp. 39–47

2. *Bergquist N R & Schilling W G K E.* Preparation of anti-human immunoglobulin for indirect fluorescent tracing of auto-antibodies. In Holborow E. J (Ed.): Standardization in Immunofluorescence. Blackwell Scientific Publ. Oxford, England 1970 pp. 171–176

3 *Beutner E. H., Holborow E. J & Johnson G D.* Quantitative studies of immunofluorescence. Immunology 13 327–337 1967

4 *Beutner E. H Sepulveda M R. & Barnett E. V* Quantitative studies of immunofluorescent staining Relationships of characteristics of unabsorbed antihuman IgG conjugates to their specific and non-specific staining properties in an indirect test for antinuclear factors. Bull. Wld Hlth Org. 39 587–606 1968.

5 *Caspersson T Zech, L. Modest E J Foley G E., Wagh U & Simonsson E.* Chemical differentiation with fluorescent alkylating agents in Vicia Faba metaphase chromosomes. Exptl. Cell. Res. 58 128–140 1969

6 *Cherry W B.* Fluorescence emission with special reference to standardization in immunofluorescence. In Holborow E. J (Ed.) Standardization in Immunofluorescence. Blackwell Scientific Publ. Oxford, England 1970 pp. 127–136.

7 *Espmark J A Grandien M & Bergquist N R.* Quantitative evaluation of direct and indirect immunofluorescence tests for viral antigens. In Beutner E. H. (Ed.) Defined Immunofluorescent Staining. Ann. N Y Acad. Sci. 177 New York 1971 pp. 98–110.

8. *Goldman M* Fluorescent Antibody Methods. Academic Press, New York and London, 1968, pp. 82–83 159

9 *Gornall, A G., Bardawill D J & David M M* Determination of serum proteins by means of the Biuret reaction. J Biol. Chem. 177 751–766 1949

10. *Hale W L & Bergquist N R.* Chessboard analyses with antinuclear antibodies. In Beutner E. H. (Ed.) Defined Immunofluorescent Staining. Ann. N Y Acad. Sci. 172 New York 1971 pp. 354 360.

11 *Kabat E. A. & Mayer M M.* Experimental Immunochemistry 2nd ed., Charles C. Thomas, Publisher Springfield, Ill. 1961 pp. 704–709

12. *Karlsson K A. & Thal, E.* Evaluation of the immunofluorescent reagents in the identification of Salmonella bacteria. Zbl. Bakt. Hyg., I Abt. Orig. A. 228, 474–481 1974

13 *Karlsson K. A., Thore A Caspersson T & Kudynowski J.* Quantitative studies of immunofluorescence in bacteria. Försvarsmedicin, Stockholm 10 61–70, 1974

14 *Kauffmann F* The Bacteriology of Enterobacteriaceae. Munksgaard, Copenhagen, 1969 pp. 86–94

15 *Kaufman, G I Nester J F & Wasserman D E.* An experimental study of lasers as excitation sources for automated fluorescent antibody instrumentation. J Histochem. and Cytochem. 19 469–475 1971

16. *Knapp W & Thal E.* Die biochemische Charakterisierung von Yersinia enterocolitica (Syn. Pasteurella "X") als Grundlage eines vereinfachten O-Antigenschemas. Zbl. Bakt. Hyg., I Abt. Orig. A 223 88–105 1973

17 *McKinney R M., Spillane J T & Pearce G W.* Fluorescein diacetate as a reference color standard in fluorescent antibody studies. Anal. Biochem. 9: 474–476 1964

18. *Mollaret H H & Thal E.* Chapter on Yersinia. In Bergey's Manual of determinative bacteriology Eighth edition. 1974

19 *Nairn R. C* Fluorescent Protein Tracing. E. and S. Livingstone Ltd Edinburgh and London, 1969 pp 303–304

20 *Pittman, B., Hebert G A Cherry W B & Taylor G C* The quantitation of nonspecific staining as a guide for improvement of fluorescent antibody conjugates. J Immunol. 98 1196–1203 1967

21 *Taylor C E. D., Heimer G V & Lidwell O M* Use of a fibre optic probe for quantitative immunofluorescence studies. Lancet, i. 785–786 1971

22 *Taylor C E. D & Heimer G V* Measuring immunofluorescence emission in terms of standard international physical units. J Biol. Standardization 2 11–20, 1974

23 *Wahren B.* Quantitative immunofluorescence of EB virus infected cells. In Beutner E. H. (Ed.) Defined Immunofluorescent Staining. Ann. N Y Acad. Sci. 177 New York 1971 pp. 113–120.

24 *Wells A F Miller C. F & Nadel M K* Rapid fluorescein and protein assay method for fluorescent-antibody conjugates. Appl Microbiol. 14 271 2 3 1966.

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ESCHERICHIA COLI SEROGROUPS IN BREAST-FED AND BOTTLE-FED INFANTS

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Ørskov, F. & Sorensen, K. Bierring. *Escherichia coli* serogroups in breast-fed and bottle-fed infants. Acta path. microbiol. scand. Sect. B, 83: 25–30, 1975.

1) Eighty-seven breast-fed and 95 bottle-fed infants were examined for differences in *Escherichia coli* serogroup pattern. 2) The serogroup composition was less complex in breast fed infants. Fewer serogroups were found in the individual breast-fed child. However, with few exceptions, the same O groups were found in the two groups of infants. The prevalent O groups were among those also found most frequently in faeces from adults. 06 strains were more common in the breast-fed group, while 07 and 016 were not at all found in that group. 3) A special search for *E. coli* strains with Kl antigen showed that this antigen predominated in the bottle-fed infants. 4) *Klebsiella* and other *Enterobacteriaceae* were more frequently found among bottle fed infants. 5) It is concluded that the intestinal milieu exerts a selective pressure which may result in a selection of different serotypes in two different intestinal milieus such as those examined.

Key words: *Escherichia coli* serogroups; breast-fed infants; bottle-fed infants.

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Many papers have been published since the beginning of this century in which the bacterial flora of breast fed and bottle fed infants has been compared. Two groups of bacteria, the bifido bacteria (*Lactobacillus bifidus* or *Bifidobacterium bifidum*) and the *Enterobacteriaceae*, and among these particularly *Escherichia coli*, have attracted special interest in such examinations. Until recently it has been generally accepted that *Bifidobacterium bifidum* was of specific importance and perhaps responsible for the qualities of the unique biochemical conditions in the intestinal lumen of the breast fed infant. A simple physical and olfactory examination of faeces from breast fed infants will give the impression that the physico-chemical milieu in the colon of the breast fed infant must be strongly influenced by the breast-milk. Many detailed examinations throughout the years have also confirmed that such chemical differences do exist, and we would like to give prominence to the brilliant examinations published recently by *Bullen & Willis* (1971) and *Bullen et al.* (1973). The findings obtained by those authors suggest strongly that *Lactobacillus bifidus* (*Bifidobacterium bifidum*) is not the cause of the special condition in the intestine of breast fed infants, but rather that the intestine of the breast fed infants offer a milieu which selects this bacterium.

The aim of this study has been to establish

to what extent differences in intestinal milieu would exert selective pressures on different *E. coli* serotype strains. In fact, merely the inadequate knowledge about the normal human intestinal flora would justify such investigations.

METHODS

Infants selected. 184 healthy infants living in their private homes, 87 breast-fed and 95 bottle-fed under three months of age were included in the investigation. Practically all of them were born in maternity homes or wards. One faeces specimen from each child was examined. The specimens were collected by health visitors who, under the auspices of the municipal authorities, offer free help and advice to all mothers in Copenhagen. The health visitors also made observations and recordings concerning growth and diet which could be of significance for the study

Bacteriological examination. 5 to 15 g samples from fresh faecal specimens without any admixture were collected. Inoculation was carried out on 14 cm diameter bromothymol blue lactose agar plates (BTB) for the examination of colony-forming *Enterobacteriaceae* In order to isolate 10 randomly selected colonies without being biased by colony appearance a number of incidentally drawn straight lines were made on the back of the plate. Ten colonies were then selected by taking all colonies crossed by one line and after that those crossed by another line until 10 colonies were isolated. The 10 colonies were transferred to a new BTB plate. This plate was inspected next day and if different cultures from one specimen looked similar they were primarily judged to belong to the same clone. According to this principle, the 10 cultures would be given, for example, the following numbers 1a, 1b, 1c, 1d, 2a, 2b, 2d, 3a and 4a. In this specimen four cultures had one appearance, four other another appearance, and finally two colonies each had their own appearance. One culture representing each colony type was selected for closer serological examination, i.e. in the above example cultures 1a, 2a, 3a and 4a. Both cultures were heated to 100° C for 1 hour followed by agglutination examination in the 157 O group sera available. For further technical details, see *Kauffmann* (1954) After O group determination of e.g culture 1a, 100 C broth cultures of 1b, 1c and 1d were examined in the O test serum to which 1a was found to belong. Confirming earlier experiences (*Orskov* 1956) the four cultures were in practically all cases found to be identical. Further examination of H antigens

TABLE 1 *Homogeneity of Faecal* Enterobacteriaceae *Flora in Breast-fed and Bottle-fed Non-hospitalized Healthy Infants*

Number of identical colonies per infant	87 breast-fed infants Number	Per cent	95 bottle-fed infants Number	Per cent
10	49	57	30	32
9	16	18	18	19
8	8	9	11	12
7	7	8	9	9
6	4	5	11	12
5 or less	3	3	16	17
Number of clones per infant				
1	49	57	30	32
2	26	30	37	39
3	11	13	16	17
4	1	1	8	8
5	0	0	4	4

10 colonies selected at random from each specimen were examined
Identity of colonies was determined by serological analysis in the case of *E. coli* and *Klebs II* and by analysis of fermentation pattern in the case of other species.

Statistical analysis
Upper part of table (colonies) $\chi^2 = 16.80$ $f = 5$ 0.1 per cent$< P <$0.5 per cent.
Lower part of table (clones) figures for 4 and 5 clones per infant taken together $\chi^2 = 18.10$, $f = 3$ $P < 0.05$ per cent.

and of biochemical and fermentative properties was carried out for each colony type on one culture only

Determination of the K1 antigen. Serological determination of this polysaccharide capsule antigen was carried out partly by slide agglutination and partly by the serum agar technique developed by *Bradshaw et al.* (1971). Rabbit OK sera produced with the two alternating forms of this antigen, K1 and K1 were used for slide agglutination (*Orskov et al.* 1971). Furthermore, special horse serum was included. This was produced by *John B. Robbins* NIH, Washington D.C., by immunization with *Neisseria meningitidis* type B bacteria. This serum, which cross-reacts strongly with K1 containing *E. coli* strains of both K1 and K1 forms was used for the serum agar plate technique. It was kindly placed at our disposal by Dr *Robbins*

RESULTS

Without exception, the faeces from the bottle fed group was foul-smelling and gray brown coloured, while faeces from the breast fed infants had an intense yellowish-green colour and a not unpleasant aromatic, butter milk-like smell.

Table 1 shows some calculations based on counting of colonies belonging to different 0 groups in the 87 breast-fed and 95 bottle fed infants. It is evident that the coli flora is more homogeneous in breast fed infants, and thus that fewer 0 groups will be found in a certain number of faecal specimens from such children. The calculations have been based on the number of identical colonies per infant and on the number of clones, and in both cases there is a significant difference between the distribution in the two groups of children.

Prevalence of Single 0 Groups

Table 2 shows in more detail the composition of the gram-negative flora in the two groups. Again it is observed that the total number of *E. coli* 0 groups is smaller among the breast fed infants, and also that fewer *Klebsiella* and other *Enterobacteriaceae* are found in that group. By and large the prevalence of the single 0 groups among the infants is very similar in the two groups.

However there are some significant differences. 0 group 6 seems to be significantly more common among the breast fed infants, while 016 and 07 which rate among the nine most frequently encountered 0 groups in the bottle-fed group, are not found at all in the breast fed group. Only in the case of 07 is the difference significant at the 5 per cent significance level. *Klebsiella* is more seldom found in the breast fed group but if present, apparently tends to constitute a larger part of the colonies. This is in contrast to the group "other *Enterobacteriaceae*" of which the number is only small if present in the breast fed infants.

A general K antigen determination has not been carried out, as this would only be of significance in this context if performed on a much larger number of infants. However because of the very special interest which at present is centred round the most frequent *E. coli* K antigen, K1 because of its close association with neonatal coli meningitis (*Robbins et al.* 1974) a special search for that antigen was carried out.

Occurrence of K1 Antigen

It will be seen from Table 3 that the number of K1-containing strains was significantly higher in the bottle-fed than in the breast fed group. The 0 groups in which K1 was found were those ordinarily associated with K1 i.e. 01 02, 07 016 083 and a few others more rarely seen. Except for 07 and 016 which, as shown in Table 2 are found only in the bottle-fed group, no difference could be found in the distribution of 0 groups associated with K1. Five infants with 07 K1 or 016 K1 were found in the bottle-fed group but even if these were excluded from this group the number of K1 strains was significantly higher in bottle fed infants.

Biochemical Examinations

Representatives of all clones isolated were examined for fermentation of several carbohydrates, viz. glucose, mannitol, sucrose, adonitol, sorbitol, arabinose, xylose rham-

TABLE 2. *Prevalence of* E. coli *O Groups and other Enterobacteriaceae in Faeces from Breast-fed and Bottle fed Infants*

E. coli O groups	87 breast fed infants Number of infants	colonies	95 bottle-fed infants Number of infants	colonies
O2	14	112	20	121
O4	8	66	9	73
O18ac	6	46	9	42
O1	4	29	8	34
O8	8	42	7	24
O7	0	0	6	23
O16	0	0	5	37
O25	4	23	5	17
O6	12	96	4	39
O5	1	9	4	25
O9	3	21	4	12
O15	4	21	4	21
O83	2	12	4	29
O22	2	15	3	14
O75	7	41	3	27
O76	0	0	3	20
O77	1	9	3	12
O117	0	0	3	14
O18ab	1	3	2	16
O88	0	0	2	13
	23 further O groups from 28 infants represented by 10 colonies or less		27 further O groups from 31 infants represented by 10 colonies or less	
Not O1 to O157	9	41	12	35
Spontan. agglutin.	11	44	6	10
Klebsiella	8	38	20	55
Other enterobact.	6	8	15	63

Statistical analysis of the significance at the 5 per cent level of differences in the prevalence of some *single O groups and other groups of bacteria*

O group 6 P = 4.2 per cent.
O group 16 P = 7 4 per cent.
O group 7 P = 3 7 per cent.

Spont. agglut. 15 per cent $<$ P $<$ 30 per cent.
Klebsiella 2.5 per cent $<$ P $<$ 5 per cent.
Other enterobact. 0 25 per cent $<$ P $<$ 0.5 per cent.

nose, raffinose, salicin, inositol and sorbose. No single fermentation reaction or pattern characterized one group of infants as compared with another. Quite a few lactose negative or lactose late positive *Escherichia* were observed and it was calculated that 20 per cent of the breast-fed and 19 per cent of the bottle-fed infants contained lactose delayed positive clones.

TABLE 3. *Prevalence of K1 Antigen in* E. coli *Strains from Faeces of Breast-fed and Bottle-fed Infants*

87 breast-fed infants	95 bottle-fed infants
10	25

Statistical analysis of the significance at the 5 per cent level of differences in prevalence of strains with K1 antigen
P = 1 77 per cent.

DISCUSSION

Recent papers by *Bullen & Willis* and associates (1971, 1973) have thrown fresh light on several conventional ideas about the conditions in the lumen of the lower intestine. They confirm that faeces of breast fed infants is dominated by anaerobic lactobacilli and that the gram-negative flora consists of smaller numbers of *Escherichia coli*. In contrast, faeces from bottle-fed infants have high numbers of *E. coli* but also other *Enterobacteriaceae* and comparatively few lactobacilli. Furthermore, these authors show that faeces from breast-fed infants has a higher water content and a lower buffering capacity than that from bottle-fed infants and that fermentable substances are found only in the faeces of breast fed infants. They suggest that the high lactose, low protein and low buffering capacity of breast milk are responsible for the selection of lactobacillus organisms in the physico-chemical milieu in the colon. They explain convincingly why breast-milk favours lactobacilli, while cows milk determines a milieu which is unfavourable to lactobacilli. *Bullen et al.* (1972) show that lactoferrin is found in comparatively large amounts in human milk, and suggest that lactoferrin in combination with specific antibody causes primary inhibition of *E. coli* in the small gut.

The present investigation confirms the findings by *Bullen & Willis* (1971) that the faecal flora of breast fed infants is more uniform, in the sense that fewer bacterial species are found, but in addition it shows that this higher uniformity also applies to the single *Escherichia* serological groups. Most 0 groups were found with frequencies which did not deviate significantly in the two groups, though differences were found in some cases. 0 group 6 was found more frequently in the breast-fed, while 07 (and 016) were more frequent in the bottle-fed infants. Furthermore, it was shown that strains with coli K1 antigen were more common among the bottle-fed infants.

The coli strains came from babies living in homes randomly distributed in the Copenhagen area and local differences in the coli flora can therefore not explain the differences (*Grüneberg et al.* 1968).

Thus, though no simple explanation of the findings can be found it has been shown that the different conditions in the intestine of the breast fed and the bottle fed infant will exert different selective pressures on some *E. coli* strains with special 0 or K antigens with consequences for the prevalence of these special serotypes in the two types of infants.

Willis et al. (1973) assume that the breast fed infant is more consistently and continuously exposed to intestinal bacterial contaminants. This suggestion does not offer any help in explaining the less complex composition of the flora of the breast-fed infants. Probably the common serogroup strains found have special capsular polysaccharide K antigens (*Ørskov et al.* 1971) which might make them less susceptible to the action of some immunological mechanisms like the lactoferrin (*Bullen et al.* 1972). On the other hand, because of the ubiquity of these common 0 groups in human subjects in all age groups, breast milk will very likely contain antibodies against precisely these 0 groups.

The K1 antigen has gained a special position recently because of its high prevalence in cases of neonatal coli meningitis. More strains containing K1 were found in bottle-fed infants, a fact which might suggest that immunological forces were involved in the selection processes working in the intestinal milieu. The distribution of other K polysaccharide antigens was not examined as they in contrast to K1 could be expected to be found only in very few infants, thereby making a sensible comparison of the frequency in the two materials impossible.

Further investigations along these ecological lines are planned, and *Willis et al.*'s success in the production of a breast-milk substitute (1973) and in carrying out simple *in-vitro* continuous growth experiments makes it tempting to try in similar experiments to imitate the conditions in the intestine of infants with diarrhoea. The aim would be to see whether a simple ecological

explanation could be found for the selection of the special serotypes found during diarrhoeal diseases both in human infants and in young animals. Recent investigations make it probable that most, if not all, such enteropathogenic strains produce plasmid-determined exotoxins, but also stress that, in addition to toxin production, the ability to colonize the intestine is a necessity. In fact, the present investigation was carried out with the view of confirming an old idea which had occurred to one of the authors namely that the biochemical milieu in the gut was of importance for the selection of special enteropathogenic serotypes (Ørskov 1952).

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REFERENCES

Bradshaw M. W. Schneerson R., Parke J. C. & Robbins J. B., Bacterial antigens cross-reactive with the capsular polysaccharide of *Haemophilus influenzae* type b. Lancet *1* 1095–1097 1971

Bullen C. L. & Willis A. T. Resistance of the breast-fed infant to gastroenteritis. Brit. med. J *3* 338–343 1971

Bullen C. L. Willis A. T. & Williams K. The significance of the bifidobacteria in the intestinal tract of infants. In Sykes, G & Skinner F. A. (Eds) Society for Applied Bacteriology Symposium Series No. – Academic Press, London 1973 p. 311–325

Bullen J. J., Rogers H. J. & Leigh, L. Iron-binding proteins in milk and resistance to *Escherichia coli* infection in infants. Brit. med J *1*: 69–75 1972.

Grüneberg, R. N. Leigh D. A. & Brumfitt W.: *Escherichia coli* serotypes in urinary tract infection. Studies in domiciliary antenatal and hospital practice. In O'Grady F. & Brumfitt, W. (Eds.) Urinary Tract Infection, University Press, Oxford 1968 p. 68–79

Kauffmann F. Enterobacteriaceae 2nd ed. Munksgaard, Copenhagen 1954 p. 169–171

Ørskov F.: Antagonism between certain coli strains isolated from cases of infantile diarrhoea and some other coli types. Acta path. microbiol. scand. Suppl. 93 *21* 239–247 1952.

Ørskov F. Escherichia coli strains isolated from cases of infantile diarrhoea and from healthy infants. Acta path. microbiol. scand. *39* 137–146 1956.

Ørskov F. Ørskov I. Jann, B. & Jann, K.. Immunoelectrophoretic patterns of extracts from all *Escherichia coli* O and K antigen test strains. Correlation with pathogenicity Acta path. microbiol. scand. Sect. B *79* 142–152, 1971

Robbins J. B., McCracken G. Gotschlich, E. Ørskov F. Ørskov I. & Ginsburg, C.: *Escherichia coli* K1 capsular polysaccharide associated with neonatal meningitis. New Engl. J Med. *290* 1216–1220, 1974

Willis A. T., Bullen C. L., Williams K., Fagg, C. G. Bourne A. & Vignon M. Breast milk substitute A bacteriological study Brit. med. J *4* 67–72, 1973

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THE ESTABLISHMENT OF K99, A THERMOLABILE, TRANSMISSIBLE *ESCHERICHIA COLI* K ANTIGEN, PREVIOUSLY CALLED "Kco", POSSESSED BY CALF AND LAMB ENTEROPATHOGENIC STRAINS

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Ørskov I Ørskov F Smith, H. Williams & Sojka, W J The establishment of K99 a thermolabile, transmissible *Escherichia coli* K antigen, previously called "Kco possessed by calf and lamb enteropathogenic strains. Acta path. microbiol. scand. Sect. B, 83 31–36, 1975

The transmissible antigen in enteropathogenic *E. coli* strains from calf and lamb, previously called Kco, is established as the *E coli* K99 antigen. It is probably of protein nature since it is destroyed by heating. It is pointed out that other antigens present, growth medium and unknown factors are of great importance for the demonstration of this antigen.

Key words *Escherichia coli* K antigen, K99 calf lamb.

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The plasmid controlled K88 antigen possessed by enteropathogenic *E. coli* pig strains was described by *Ørskov et al.* (5 6) and was shown to be a protein by *Stirm et al.* (12) In 1971 *Smith & Linggood* (11) reported on another transmissible K antigen from *E. coli* calf and lamb strains. This antigen was referred to for convenience, as the "common K antigen" = Kco. The antigen was originally discovered by *Sojka* (11) who had observed that many calf and lamb enteropathogenic strains, although having different O antigens, possessed a cross-reacting K antigen. Some such strains were received at the WHO Collaborative Centre for Reference and Research on Escherichia for further examination, particularly of the Kco antigen. The present paper is a report of the serological studies of this antigen.

MATERIAL AND METHODS

The Kco antigen studies were based on examination of seven strains isolated from calves and four from lambs. The strains were submitted by *W J Sojka* with the information that they had been

isolated from epidemiologically unrelated cases of diarrhoea. They had all dilated ligated segments of calf and lamb intestine and they had all produced diarrhoea when given orally to calves and lambs (10). Many confusing serological results had been obtained with these strains since most of them reacted not only with homologous but also with heterologous OK antisera in slide agglutination tests, suggesting that they might share one or more common antigens. With one exception, the O antigens of the strains had been serologically determined.

Production of antisera, methods for adsorption and agglutination tests were performed as described by *Kauffmann* (2). The techniques employed for precipitation tests were double diffusion in gel (7) and immunoelectrophoresis (4, 8). Direct haemagglutination of guinea pig red cells was used as described previously (13).

Beef broth was used for fluid and solid media (7), the latter denoted BA. A trypticase glucose (0.05 per cent) extract agar medium (9), called TGXA, was also used as solid medium.

RESULTS

General Serological Determination

Overnight broth cultures heated at 100° C for 1 hour were tested for agglutination by the available antisera 01–0157. The previous O antigen diagnosis of the strains was confirmed. The strain whose O antigen had not been determined was found to possess 09.

TABLE 1. *Serotypes of Strains Examined*

Strain designation	Serotype O	K	H	Isolated from
B44	9	30	–	Calf
B117	8	65	–	Calf
B42	9	35	–	Calf
B85	9		–	Calf
B79	101	32	–	Calf
B41	101		–	Calf
B111	101		–	Calf
S13	8	85	?	Lamb
S14	8	85		Lamb
S15	8	85		Lamb
S16	8	85	?	Lamb

Serotype means result of examination of O (lipopolysaccharide), K (polysaccharide) and H (protein) antigens. Thus the K99 antigen is not included in the serotypes here.

? = not sufficiently motile for H antigen determination.

By slide agglutination tests followed by immunoelectrophoresis and double diffusion in gel, the K antigens were determined. The results are shown in Table 1. The double diffusion test did not show complete identity with the test K antigens. One 09 strain and two 0101 strains were recorded as K–. Only lines on the cathodic side of the application well were seen when these strains were examined in immunoelectrophoresis with homologous O or OK antiserum, i.e. no K antigen which could be separated from the O antigen could be demonstrated.

All strains isolated from calves and two lamb strains were non-motile. The two remaining lamb strains were too weakly motile for their H antigens to be determined.

Examination of the Transferable Antigen

All 11 strains were examined for Kco antigen by slide agglutination tests with OK antisera prepared against four calf strains and one lamb strain. Positive reactions were seen both with homologous and heterologous antisera, but in a peculiar way. The intensity of the heterologous reaction not only differed from strain to strain but also from test to test. It was then realised that the reaction was dependent on the K (polysaccharide) antigen state and the medium. The strain which varied least was B41 in which no polysaccharide K antigen had been detected. The OK antiserum against this strain was depleted of O antibodies by absorption with homologous boiled culture; agglutinins were however still found reacting with live culture. The agglutinates were loose and almost disappeared after shaking. This absorbed antiserum was used as test serum for demonstration of the Kco antigen in the other strains. In the present studies, this antigen was most easily demonstrated in the more transparent colonies, i.e. those with the least amount of K antigenic polysaccharide present. TGXA was better than BA medium. A summary of these results is given in Table 2. This shows that all cultures except S14 and S15 could be made to react with the absorbed

TABLE 2. *Result of Slide Agglutination Tests of Non-transparent and Transparent Colonies from BA and TGXA Plates in B41 OK Antiserum Absorbed by Homologous 100° C Culture*

Non-transparent colonies of	Medium BA	Medium TGXA	Transparent colonies of	Medium BA	Medium TGXA
B44	—	+ + +	B44	—	+ + +
B117	—	—	B117	—	+ + +
B42	+ +	+ +	B42	+ +	+ + +
n.e.			B85 white	+ + +	+ + +
n.e.			B85 grey	—	—
B79	—	+ +	B79	+ +	+ + +
n.e.			B41	+ +	+ + +
n.e.			B111	+ +	+ + +
S13	—	—	S13	+ + +	+ + +
S14	—	—	S14	—	—
S15	—	—	S15	—	—
S16	+ +	+ +	S16	+ + +	+ + +

— means no agglutination.
+ + + and + + express a strong and less strong degree of agglutination.
n.e. means not existing.

TABLE 3 *Result of Tube Agglutination Tests in B111 OK Antiserum Absorbed in Different Ways*

Antigen	Antiserum OK B111 Unabs.	Abs. by 100° C** homolog. culture	Further abs. by B85 white non-heated	B85 white 100 C	B85 white non-heated grown at 18° C	B85 grey non-heated
B44	2560	1280	0	2560	1280	2560
B117	640	1280	0	640	1280	1280
B42	1280	2560	0	5120	1280	1280
B85 white	5120	1280	0	2560	1280	1280
B85 grey	0	0	0	0	0	0
B41	2560	1280	0	2560	640	1280
B111	2560	1280	0	640	640	640
S13	320	320	0	320	320	640
S16	640	640	0	640	640	640

All cultures were grown on TGXA medium and at 37 C except where indicated.
0 means titre <20. 5120 means ≧5120.
With the exception of B41 and B111 (both containing O101 antigen) no 100° C culture agglutinated in this antiserum.
** No agglutination of 100° C cultures of B41 and B111 was seen in the absorbed antiserum.

antiserum. Addition of 0 1 per cent glucose to BA or omission of glucose from TGXA medium did not influence the reactions.

After growth at 18 C, the Kco antigen was not demonstrable, shown by the fact that such cultures neither agglutinate nor have any agglutinin-binding capacity The same was true of cultures grown at 37 C and then heated to 100 C for 1 hour Some of these facts are shown in Table 3 This also shows that B85 can be separated into a white and a grey variety and that the grey has lost the

TABLE 4 *Identity of K99 Antigen of B41 and B85 Demonstrated in Tube Agglutination Test*

Antigen		Antisera OK B41 Unabs.	Abs. by 100 C homolog. culture	Further abs. by B85 non-heated	Further abs. by B117 non-heated	Antisera OK B85 Unabs.	Abs. by 100° C homolog. culture	Further abs. by B41 non heated	Further abs. by B117 non-heated
Non-heated	B44	5120	5120	0	20	5120	5120	0	40
	B117	5120	5120	0	20	1280	2560	0	0
	B42	5120	5120	0	20	5120	5120	0	80
	B85 white	5120	5120	0	0	5120	5120	0	0
	B79	5120	5120	0	0	5120	5120	0	0
	B41	5120	5120	0	20	5120	5120	0	20
	B111	5120	5120	0	20	5120	2560	0	80
	813	2560	2560	0	0	2560	1280	0	0
	816	5120	5120	0	0	2560	2560	0	0
100° C	B41	5120	0	/	/	0	0	/	/
	B85	20	20	/	/	5120	0	/	/

See key to Table 3

Kco antigen, since it does not agglutinate in the B111 antiserum and is incapable of removing Kco antibodies.

The Kco antigen was demonstrated in nine of the 11 strains and identity between the Kco antigen og B85 and B41 was proved, as shown in Table 4. Antiserum against B117 was also available but could not be absorbed in the same manner because of a low content of Kco antibodies.

The Kco antigen is established as K99 and strain B41 will be the test strain as it is most easy to demonstrate the antigen in this strain.

Attempts to demonstrate the K99 antigen in immunoelectrophoresis or in gel precipitation test were unsuccessful. It is noteworthy that the K88 antigen is difficult, though not impossible, to demonstrate by these methods.

In a direct haemagglutination test for demonstration of fimbriae, carried out in the cold with guinea pig red blood cells, some of the strains possessing the K99 antigen were positive after 5–10 minutes, the reaction not being inhibited by mannose. Some were negative or not positive until after 30–40 minutes, in which case the reaction was highly doubtful and was recorded as negative. It is thus an unanswered question whether the presence of K99 causes a mannose-resistant haemagglutination like the K88 antigen did (13).

DISCUSSION

The aim of the present paper was to give a detailed serological account of a transmissible antigen possessed by enteropathogenic calf and lamb strains. This antigen, previously called Kco, is now designated K99 (the last established K number was K94 but publications on K95–K98 are in preparation). Presumably it consists of protein, like K88.

The K99 antigen may be difficult to demonstrate with certainty as this depends on other antigens present, on growth medium and on unknown factors. It was, however, demonstrated in nine of the 11 strains examined. For the reasons mentioned above it may well be that the two remaining strains also contained K99. As the conditions for its demonstration cannot be defined with certainty one could say that it is too early to give the antigen an internationally established number. However, there is a close association between possession of this antigen and enteropathogenicity for calves and lambs. Also *Smith & Linggood* (11) have found that $K99^+$ forms proliferate in the small intestine while K99 forms do not, suggesting that the K99 antigen may function by facilitating adhesion to intestinal calf and lamb epithelium similar to the function of the K88 antigen in some pig enteropathogenic strains. For this reason it was found of importance to publish additional knowledge of the K99 antigen.

The O8 strains dealt with in the present paper contain a K antigen related to K85. The polysaccharide antigen, K85, was originally characterized as the K antigen in strains with antigen O141 (3). Later results with immunoelectrophoresis (4) and chemical analysis (*B. Jann & K. Jann*, personal communication) indicated that the O141 K85 strain possesses an acidic lipopolysaccharide and no special acidic K polysaccharide which can be separated from the lipopolysaccharide. This finding fits well with the fact that antigen O141 is always found together with the so-called K85 antigen. The basis of the establishment of K85 was the inagglutinability of the living strain in the homologous O antiserum. The final explanation for O inagglutinability has not yet been found, but we know that it can be caused by *e.g.* a polysaccharide K antigen, flagella or fimbriae.

A situation possibly similar to that of O8 K85 and O141:K85 strains has been proved chemically concerning the swine strain 145, the test strain for K antigen 87 (1). The serotype of this strain was specified as (O32) K87:H45 (5). This strain has no special K antigen and that which we called K87 is determined by the lipopolysaccharide side chain, *i.e.* the O antigen which is related to antigen O32. However, in another strain, D227, a $K88^-$ form of the swine strain, G7 with the serotype O8:K87:H19, the K87 de

TABLE 4 Identity of K99 Antigen of B41 and B85 Demonstrated in Tube Agglutination Test

Antigen		Antisera: OK B41 Unabs.	OK B41 Abs. by 100° C homolog. culture	OK B41 Further abs. by B85 non-heated	OK B41 Further abs. by B117 non-heated	OK B85 Unabs.	OK B85 Abs. by 100 C homolog. culture	OK B85 Further abs. by B41 non-heated	OK B85 Further abs. by B117 non-heated
Non-heated	B44	5120	5120	0	20	5120	5120	0	40
	B117	5120	5120	0	20	1280	2560	0	0
	B42	5120	5120	0	20	5120	5120	0	80
	B85 white	5120	5120	0	0	5120	5120	0	0
	B79	5120	5120	0	0	5120	5120	0	0
	B41	5120	5120	0	20	5120	5120	0	20
	B111	5120	5120	0	20	5120	2560	0	80
	S13	2560	2560	0	0	2560	1280	0	0
	S16	5120	5120	0	0	2560	2560	0	0
100 C	B41	5120	0	/	/	0	0	/	/
	B85	20	20	/	/	5120	0	/	/

See key to Table 3

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INFLUENCE OF METHYLPREDNISOLONE ON COMPLEMENT ACTIVITY IN GERMFREE RATS AND ON ANTIBODY ACTIVITY TO *ESCHERICHIA COLI* IN MONOCONTAMINATED RATS

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Baardsen, A. Midtvedt, T. & Trippestad, A. Influence of methylprednisolone on complement activity in germfree rats and on antibody activity to *Escherichia coli* in monocontaminated rats. Acta path. microbiol. scand. Sect. B, 83 37–44 1975

By means of radio-isotope technique, the influence of methylprednisolone treatment on opsonic and bactericidal serum activities against *E. coli* was studied on germfree and monocontaminated rats. Based on the present results it seems unlikely that methylprednisolone impairs the production of complement components. A slight suppression of antibody activity was only found in markedly wasted monocontaminated rats treated with very high doses of methylprednisolone from the day before contamination.

Key words: Methylprednisolone complement activity antibody activity germfree life; rats.

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Increased susceptibility to infections following administration of glucocorticosteroids (steroids) is a well-established fact. The drugs might affect host factors involved in the defence against microorganisms, or the microorganism itself. A direct effect on microorganisms seems unlikely since the results of a previous study (8) showed that the sensitivity of *E. coli* to rat serum was unaltered following pre-incubation of the bacteria with methylprednisolone. The opsonic and bactericidal activities of serum towards *E. coli* were blocked by methylprednisolone in concentrations exceeding 1 mg per ml, which corresponds to at least 25 times the serum concentrations obtained in clinical use.

A decreased activity of complement components has been observed in steroid treated animals (2). Whether this was due to impaired synthesis and/or increased degradation of complement components, was not stated.

It has been claimed that steroids alter the metabolism of immunoglobulins (6, 12, 13, 14). The rate of degradation is uncertain, since conflicting results are reported (6, 13). Steroids have been found to suppress the formation of antibodies to heterologous erythrocytes (3, 4, 5, 10, 12). A specific interference with the early inductive phase of the immune response has been suggested. However observations based on a single injection of such antigens hours before or after

termining sugars are part of a special acidic polysaccharide K antigen and thus not part of the lipopolysaccharide molecule. The lipopolysaccharide side chain of this strain determines the O8 antigen. If the K85 antigen is comparable to the K87 antigen, the K85 specificity will in some *E. coli* strains,like those reported in this paper be found in the acidic polysaccharide K antigen, while this same specificity in other strains hitherto called O141.K85 resides in the lipopolysaccharide side chain. The O and K antigens of the O8.K85 strains described here are being further investigated chemically

REFERENCES

1 *Jann B Jann, K., Schmidt G., Ørskov F & Ørskov I* Comparative immunochemical studies of the surface antigens of *Escherichia coli* strains O8.K87(B?).H19 and (O32): K87(B?) H45 Europ. J Biochem. *23* 515–522 1971

2. *Kauffmann F.*. The bacteriology of *Enterobacteriaceae* Munksgaard, Copenhagen, 1966.

3 *Ørskov F Ørskov I Rees T A & Sahab K.* Two new *E. coli* O-antigens O141 and O142 and two new coli K-antigens: K85 and K86 Acta path. microbiol. scand. *48* 48–50 1960.

4 *Ørskov F., Ørskov I., Jann, B. & Jann K.* Immunoelectrophoretic patterns of extracts from all *Escherichia coli* O and K antigen test strains. Correlation with pathogenicity Acta path. microbiol. scand. Sect. B, *79* 142–152, 1971

5 *Ørskov I Ørskov F Sojka, W J & Leach J M.*. Simultaneous occurrence of *E. coli* B and L antigens in strains from diseased swine. Acta path. microbiol. scand. *53* 404–422 1961

6 *Ørskov I & Ørskov F.*. Episome-carried surface antigen K88 of *Escherichia coli*. I Transmission of the determinant of the K88 antigen and influence on the transfer of chromosomal markers. J Bact. *91* 69–75 1966

7 *Ørskov I & Ørskov F.*. The K antigens of *Escherichia coli*. Re-examination and re-evaluation of the nature of L antigens. Acta path. microbiol. scand. Sect. B, *78* 593–604 1970.

8. *Scheidegger J J* Une micro-méthode de immunoélectrophorèse. Int. Arch. Allergy *7* 103–110 1955

9 *Schlecht S & Westphal O*: Wachstum und Lipopolysaccharid (O-Antigen) Gehalt von Salmonellen bei Züchtung auf Agarnährboden. Zbl. Bakt. I Abt. Orig *200* 241–259, 1966.

10. *Smith H Williams & Halls S* Observations by the ligated intestinal segment and oral inoculation methods on *Escherichia coli* infections in pigs, calves, lambs and rabbits. J. Path. Bact. *93* 499–529 1967

11 *Smith, H Williams & Linggood M A* Further observations on *Escherichia coli* enterotoxins with particular regard to those produced by atypical piglet strains and by calf and lamb strains The transmissible nature of these enterotoxins and of a K antigen possessed by calf and lamb strains. J Med. Microbiol. *5* 243–250. 1971

12. *Stirm S Ørskov F., Ørskov I & Mense, B.* Episome-carried surface antigen K88 of *Escherichia coli* II Isolation and chemical analysis. J Bact. *93* 731–739 1967

13 *Stirm S., Ørskov F Ørskov I & Birch-Andersen A.*. Episome-carried surface antigen K88 of *Escherichia coli*. III Morphology J Bact. *93* 740–748, 1967

−12, and −1 respectively and thereafter once daily for 12 days. Calculated on the basis of post treated weights recorded after removal of rats from isolator this dosage ranged approximately from 50 to 100 mg per kg per day during the treatment period.

Serum

Twenty four hours after the last medication the rats were removed from the isolators and blood collected by heart puncture. Sera from rats within the individual groups of each series were pooled and stored at −20° C.

Inactivation of serum was obtained by heating at 56 C for 30 minutes (7) prior to antibody titration.

Complement

Serum from GF rats was used as a source of complement (7)

Determination of Complement Activity

of sera from GF rats and GF rats treated with methylprednisolone (GFU) was performed after addition of 10 per cent inactivated conventional rat serum as a standard amount of antibody (7)

Determination of Antibody Activity

of inactivated sera was performed after addition of 5 per cent GF serum as a standard amount of complement (7)

Determination of Immunoglobulin Class

was based on mercapto-ethanol (ME) treatment of serum and subsequent alkylation by iodacetamide as previously described (7)

Determination of Phagocytosis and Release of Label from Bacteria into Medium

was carried out as previously described, using 15 min as a standard period of incubation at 37 C (7 18) The opsonic activity of serum was studied by measuring its ability to induce uptake of ^{32}P-labelled *E. coli* into polymorphonuclear neutrophils (PMN) which were suspended in medium containing 5 per cent of the serum to be tested. The uptake of radioactivity was related to cell protein (18)

Bactericidal activity of serum was measured indirectly using release of label from bacteria into the medium as parameter

Statistical Analysis

was carried out by use of the two samples ranks test of Wilcoxon-White (13)

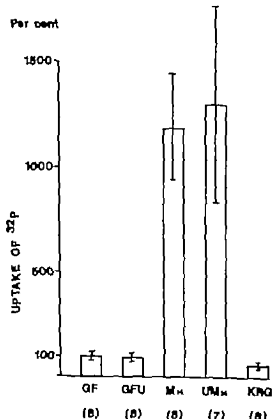


Fig 1 Phagocytosis by PMN Uptake of ^{32}P labelled *E. coli* incubated in medium without serum (KRG) and by addition of different sera, i.e. GF and GF rats, GFU from GF rats treated with methylprednisolone, M14 from ex-germfree rats monocontaminated with *E. coli* for 14 days, UM14 from M14 rats simultaneously treated with methylprednisolone. The mean uptake rate per mg cell protein in the presence of GF serum is set as 100 per cent. I = ±1 SD Brackets indicate number of observations.

RESULTS

Phagocytosis

The rates of uptake of ^{32}P labelled *E. coli* by PMN in the presence of sera from the GF1 series of rats are presented in Fig. 1 Serum from GF rats treated with methylprednisolone (GFU) induced a rate of uptake which was not significantly different ($p>0.10$) from that induced by GF control serum. Serum from the monocontaminated rats (M14) induced a rate of uptake 12 times higher than that induced by GF serum.

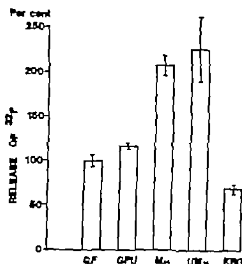


Fig 2 Release of label from 32P-labelled *E. coli* incubated in medium without serum (*KRG*) and by addition of the different sera (10 per cent) explained in legend to Fig. 1 The rate of release in the presence of GF serum is set as 100 per cent. *I* = ±*1 SD* (n = number of observations)

Serum from monocontaminated rats *simultaneously* treated with methylprednisolone (UM14) induced a rate of uptake which was not significantly different from that of the M14 control serum ($p>0.10$)

Bactericidal Activity

In the presence of 10 per cent serum from the GF1 series of rats, is presented in Fig 2. The release of label *from bacteria in the* presence of serum from monocontaminated rats (M14) doubled that induced by GF serum. As compared to the M14 control serum, the release induced by serum from monocontaminated rats simultaneously treated with methylprednisolone (UM14) showed no significant difference ($p>0.10$) The release induced by serum from GF rats treated with methylprednisolone was not reduced compared to that induced by GF serum.

Antibody Activity in Relation to Time Interval from Contamination

By titration of serum from rats monocontaminated with *E. coli* for 14 days (M14) and 28 days (M28) respectively the release of label induced by these sera showed no significant difference ($p>0.10$) However the activity of ME-resistant fraction of serum increased significantly ($p<0.01$) with the contamination period studied, as shown in Fig. 3 at dilution 1 2000.

Influence of Drug Timing in Relation to Antigenic Stimulation

The commencement of 2 weeks of methylprednisolone treatment was varied in relation to contamination with *E coli* and antibody activity of sera from treated rats and

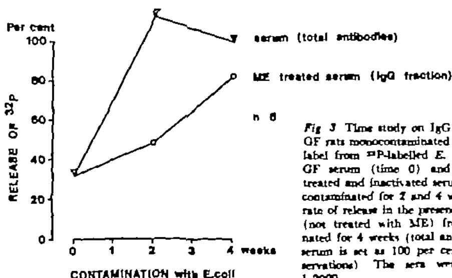


Fig 3 Time study on IgG fraction of serum from GF rats monocontaminated with *E. coli*. Release of label from 32P-labelled *E. coli* in the presence of GF serum (time 0) and by addition of ME treated and inactivated serum from GF rats monocontaminated for 2 and 4 weeks, respectively The rate of release in the presence of inactivated serum (not treated with ME) from rats monocontaminated for 4 weeks (total antibody activity) + GF serum is set as 100 per cent (n = number of observations) The sera were tested at dilution 1 2000.

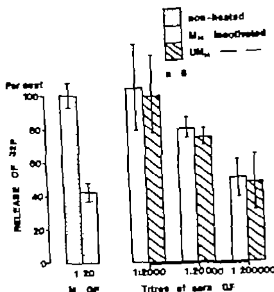


Fig 4 Release of label from ^{32}P-labelled *E. coli* incubated in the presence of different amounts of serum from GF rats monocontaminated with *E. coli* for 14 days (M14) and serum from M14 rats simultaneously treated with methylprednisolone (UM14) By dilution of these inactivated sera, 5 per cent GF serum was added. The rate of release in the presence of (not inactivated) 5 per cent serum from GF rats monocontaminated with *E. coli* for 28 days (M28) without the addition of *GF serum is set as 100 per cent* I = ±1 SD (n = number of observations)

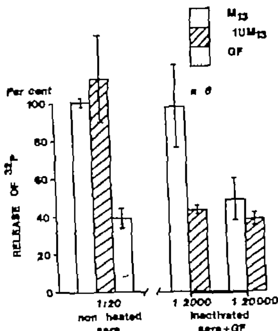


Fig 6 Release of label from ^{32}P-labelled *E. coli* in the presence of serum from GF rats monocontaminated with *E. coli for 13 days (M13) and* serum from M13 rats treated with methylprednisolone from day −1 of contamination (−1UM13) By testing dilutions of sera, GF serum was added as a source of complement The rate of release in the presence of non-heated M13 serum (5 per cent) without the addition of GF serum is set as 100 per cent. I = ±1 SD (n = number of observations)

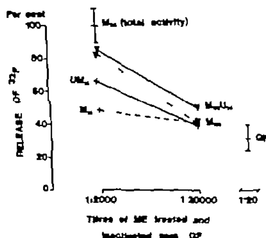


Fig 5 Antibody activity of ME-treated sera from different groups of rats explained in Table 1 The rate of release in the presence of M28 serum (not treated with ME) *is set as 100 per cent.*

untreated controls was tested. The results of drug treatment simultaneously with contamination are presented in Fig 4 By titration, the total antibody activity of sera from treated rats was not significantly different from that of controls ($p > 0.10$) Corresponding results were found when drug treatment was started 14 days after contamination. The rate of release induced by the ME-resistant *fraction* of sera from drug treated rats was not reduced in relation to that induced by the corresponding fraction of control sera (Fig 5) However pretreatment of GF rats for one day with methylprednisolone before contamination with *E. coli* resulted in a significant reduction of total antibody activity of serum compared to controls at the titre 1:2000 (*$p < 0.01$*) *as shown in Fig 6*

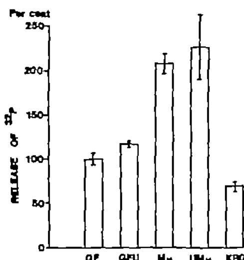


Fig 2 Release of label from 32P-labelled *E. coli* incubated in medium without serum (KRC) and by addition of the different sera (10 per cent) explained in legend to Fig 1 The rate of release in the presence of GF serum is set as 100 per cent. I = ±1 SD (n = number of observations)

Serum from monocontaminated rats *simultaneously* treated with methylprednisolone (UM14) induced a rate of uptake which was not significantly different from that of the M14 control serum ($p>0.10$)

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In the presence of 10 per cent serum from the GF1 series of rats, as presented in Fig. 2. The release of label from bacteria in the presence of serum from monocontaminated rats (M14) doubled that induced by GF serum. As compared to the M14 control serum, the release induced by serum from monocontaminated rats simultaneously treated with methylprednisolone (UM14) showed no significant difference ($p>0.10$) The release induced by serum from GF rats treated with methylprednisolone was not reduced compared to that induced by GF serum.

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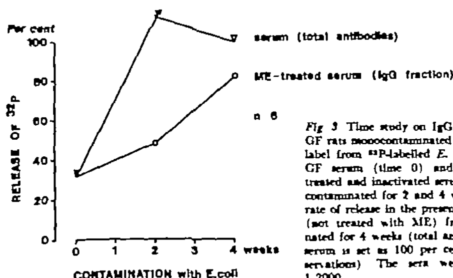


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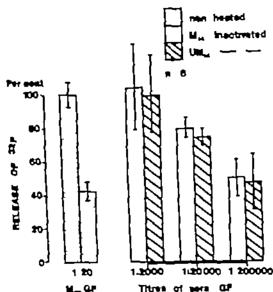


Fig 4 Release of label from ^{32}P-labelled *E. coli* incubated in the presence of different amounts of serum from GF rats monocontaminated with *E. coli* for 14 days (M14) and serum from M14 rats simultaneously treated with methylprednisolone (UM14) By dilution of these inactivated sera, 5 per cent GF serum was added. The rate of release in the presence of (not inactivated) 5 per cent serum from GF rats monocontaminated with *E. coli* for 28 days (M28) without the addition of GF serum is set as 100 per cent. I = ±1 SD (n = number of observations)

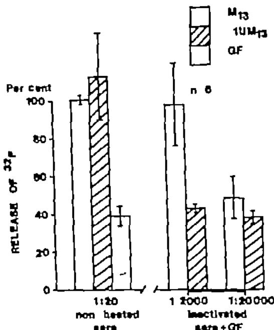


Fig 6 Release of label from ^{32}P-labelled *E. coli* in the presence of serum from GF rats monocontaminated with *E. coli* for 13 days (M13) and serum from M13 rats treated with methylprednisolone from day −1 of contamination (−1UM13) By testing dilutions of sera, GF serum was added as a source of complement. The rate of release in the presence of non-heated M13 serum (5 per cent) without the addition of GF serum is set as 100 per cent. I = ±1 SD (n = number of observations)

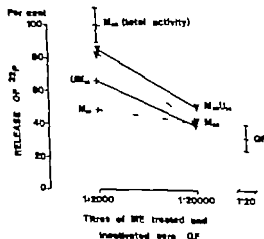


Fig 5 Antibody activity of ME-treated sera from different groups of rats explained in Table 1 The rate of release in the presence of M28 serum (not treated with ME) is set as 100 per cent.

untreated controls was tested. The results of drug treatment simultaneously with contamination are presented in Fig 4 By titration, the total antibody activity of sera from treated rats was not significantly different from that of controls ($p>0.10$) Corresponding results were found when drug treatment was started 14 days after contamination. The rate of release induced by the ME-resistant fraction of sera from drug-treated rats was not reduced in relation to that induced by the corresponding fraction of control sera (Fig. 5) However pretreatment of GF rats for one day with methylprednisolone before contamination with *E. coli* resulted in a significant reduction of total antibody activity of serum compared to controls at the titre 1:2000 ($p<0.01$) as shown in Fig 6

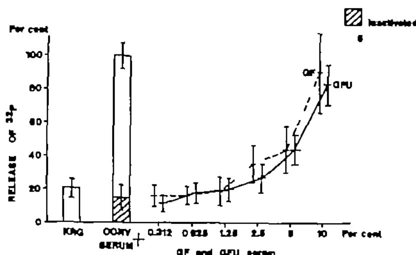


Fig 7 Release of label from ^{32}P-labelled *E. coli* suspended in medium without serum (KRG) and in the presence of serum from conventional rat serum before and after inactivation and by addition of increasing amounts of serum from GF rats and GF rats treated with methylprednisolone (GFU) I = ±1 SD

Complement Activity

Inactivated conventional rat serum (10 per cent) was used as a standard amount of antibodies. The bactericidal activity of this serum was raised by the addition of GF serum and serum from GF rats treated with methylprednisolone (GFU) as sources of complement. By titration, the GFU serum showed the same whole complement activity as the control serum (Fig 7)

Other Nonspecific Humoral Factors

The possible role played by nonspecific humoral factors apart from complement in this test system is reflected by the difference between observed uptake in the absence (KRG) and that in the presence of GF serum (without specific antibodies) (Fig 1) The corresponding difference in bactericidal activity is illustrated in Fig 2 The magnitude of these differences was 3 per cent of the uptake induced by M14 serum, 10 per cent of the bactericidal activity of M14 serum.

DISCUSSION

Atkinson & *Frank* (2) studied the effect of cortisone on the level of complement components *in vivo* using haemolytic titration as functional assay They concluded that high dose cortisone therapy produced a marked depression in the titre of a number of complement components in guinea pigs. However it was impossible to say whether altered synthetic and/or catabolic rates were responsible for that phenomenon. Probably complement consuming processes might have been going on in the animals tested, due to the infection-enhancing effect of cortisone. In this field, no *in vitro* studies of germfree animals are available. These animals, kept in a controlled environment, are protected against complement-consuming infections during steroid treatment.

The present results seem to indicate that high dose steroid administration to rats does not impair the synthesis of complement to a measurable degree in the bactericidal reaction used. The probable advantage of using complement and antibody from the same strain of animals is combined with the advantage of using fresh GF serum directly as a source of complement without purification procedures Such procedures might imply risks of interference with the activity of pertinent components.

It appears from an impressive number of

reports that the influence of steroids on antibody formation depends upon the dose of antigen and on the timing and dosage of steroid administration (10 11 12) In rats (3) and mice (5) *Berglund* observed that prednisolone suppressed the early haemolysin response to sheep red blood cells when the drug was administered 12 hours before injection of antigen. In rats, this response was significantly inhibited by seven hourly injections of prednisolone commenced 12 hours before the antigen injection. When the same treatment was started 28 hours before antigen injection, no effect was found, indicating that the function of the immunogenic tissue was rapidly restored.

In bacterial infections, the nature and dose of antigens obviously differ from those used in the above mentioned experiments with heterologous erythrocytes. Considering the vast amount of data accumulated in this field (10 12) the lack of pertinent reports on complement mediated bactericidal reactions is surprising.

Germfree rats monocontaminated with *E. coli* appear to be suitable for evaluation of drug influences on the synthesis of antibodies (7)

Establishment of a bacterial flora in the gut represents a very strong and continuous antigenic stimulation, probably resulting in a mixture of primary and secondary antibody responses after two weeks (1 7) As indicated by the present results, the total antibody activity against *E. coli* was of the same magnitude when measured two and four weeks after contamination with the test microbe, although the ME-resistant antibodies, presumably IgG seemed to increase during the observation period. Therefore steroid influence on the primary as well as the secondary (late) response might probably be evaluated in the present model.

A high-dose methylprednisolone therapy neither reduced the whole antibody activity nor the IgG activity in relation to controls, whether the administration of hormone started simultaneously with or after contamination. Pretreatment for one day with methylprednisolone, however resulted in a lower bactericidal activity of serum at titre 1:2000 in relation to controls.

However a complete depletion of lymphocytes was found in the peripheral blood of treated rats in this group (9) and their weight after treatment was 38 per cent less than that of the untreated controls. Apart from this marked wasting disease (17) these rats might also have suffered from an infection enhanced by the high dosage of methylprednisolone (50–100 mg per kg per day) Therefore, it seems likely to explain the slight reduction of antibody activity found in their sera, as a result of a nonspecific inhibition of antibody-forming cells and/or as a result of increased consumption of antibodies.

Based on the present results it seems unlikely that high-dose steroid treatment impairs the synthesis of complement in rats.

A slight suppression of antibody formation to *E. coli* was obtained when extremely high long-term dosage of methylprednisolone was started prior to antigen. When this treatment was started simultaneously with or after antigen, no impairment of antibody formation was found. The presence of a bacterial flora in wasted steroid-treated rats monocontaminated with *E. coli* (9 17) might suggest increased consumption of antibodies (13 14) If so, the maintenance of a high level of antibodies in these rats would actually indicate increased formation of antibodies to compensate for increased consumption of the latter

REFERENCES

1 *Ahlstedt S Holmgren J & Hanson L. Å* The primary and secondary antibody response to *Escherichia coli* 06 lipopolysaccharide analyses at the humoral and cellular level. Amount and avidity of the antibodies in relation to protective capacity Immunology 24 191–202, 1973.

2 *Atkinson, J P & Frank M M* Effect of cortisone therapy on serum complement components. J Immunol. *111* 1061–1066 1973

3 *Berglund, K* Studies on factors which condition the effect of cortisone on antibody production. I. The significance of time of hormone administration in primary hemolysin

response Acta path. microbiol. scand. 38 311–328, 1956.

4 *Berglund K.*, Studies on factors which condition the effect of cortisone on antibody production. 2. The significance of dose of antigen in primary hemolysin response. Acta path. microbiol. scand. *38* 329–338, 1956.

5 *Berglund K* Inhibition of antibody formation by prednisolone Location of a short sensitive period. Acta path. microbiol. scand. *55* 187–202 1962.

6. *Butler W T & Rossen R D.*, Effect of methylprednisolone on immunoglobulin metabolism in man. Fed. Proc. *32* 1028, 1973

7 *Baardsen A., Midtvedt T & Trippestad A.*, Influence of azathioprine on humoral defence factors against *Escherichia coli* in germfree and monocontaminated rats. Acta path. microbiol. scand. Sect. B, *81* 799–805 1973

8. *Baardsen A Trippestad A & Midtvedt T* In vitro influence of glucocorticosteroids on opsonic and bacterial activities against *Escherichia coli* in rat serum. Acta path. microbiol. scand. Sect. B, *82* 696–700 1974

9 *Baardsen A Midtvedt T & Trippestad A* The influence of azathioprine and methyl prednisolone on white blood cells in conventional, germfree and ex-germfree rats monocontaminated with *E. coli.* To be published.

10 *Dukor P & Dietrich, F M.*, Characteristic features of immunosuppression by steroids and cytotoxic drugs. Arch. Allergy *34* 32–48 1968.

11 *Elliott E. V & Sinclair N R. St C* Effect of cortisone acetate on 19S and 7S haemolysin antibody A time course study Immunology *15* 643–652, 1968.

12. *Gabrielsen A. E. & Good R A* Chemical suppression of adaptive immunity Advanc. Immunol. *6* 91–229 1967

13 *Griggs R C., Condemi J J & Vaughan J H* Effect of therapeutic dosages of prednisone on human immunoglobulin G metabolism. J Allergy Clin. Immunol. *49* 267 273, 1972.

14 *Levy A L. & Waldmann T A.*, The effect of hydrocortisone on immunoglobulin metabolism. J clin. Invest. 49 1679–1684 1970.

15 *Mainland D* Elementary medical statistics W B. Saunders Co., Philadelphia & London, 1964

16. *Midtvedt T & Trippestad A* Opsonizing and bactericidal effects of sera from gnotobiotic and conventional rats on ^{32}P labelled *E. coli.* Acta path. microbiol. scand. Sect. B, *78* 1–5 1970

17 *Reed N D & Jutila, J W* Wasting disease induced with cortisol acetate. I Studies in germ-free and conventionally reared mice. J Immun. *99* 238–245 1967

18. *Trippestad A & Midtvedt T* Phagocytosis of ^{32}P labelled *E. coli* by rat peritoneal polymorphonuclear leucocytes. Evaluation of a method. Acta path. microbiol. scand. *74* 259 273 1968.

Acta path. microbiol. scand. Sect. B, 83 45–47 1975

PRODUCTION OF CELL-BOUND ANTIGENS BY *BACTEROIDES FRAGILIS* NCTC 9343 IN CONTINUOUS CULTURE

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Dalland, E. & Hofstad, T Production of cell-bound antigens by *Bacteroides fragilis* NCTC 9343 in continuous culture. Acta path. microbiol. scand. Sect. B, 83 45–47 1975

Highly active antigen preparations, as measured by precipitation and inhibition of haemagglutination, were obtained from *Bacteroides fragilis* NCTC 9343 grown in continuous culture. The activity varied slightly only with the glucose concentration of the medium and the dilution rate. pH had a greater influence on antigen production.

Key words: *Bacteroides fragilis* cell-bound antigens continuous culture.

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For production of microorganisms of constant antigenic composition, qualitatively and quantitatively strict control of environment seems to be necessary (*Pirt et al.* 1961) This may be achieved by culturing the organisms in a chemostat were the rate of cell growth is dependent only on the rate of addition of fresh medium and the organisms remaining in the logarithmic phase of growth over a wide range of dilution rates (*Herbert et al.* 1956) In addition, the continuous culture technique is well suited for investigating the effect of environmental factors on antigen production.

For attainment of high cell yields, *Bacteroides fragilis* NCTC 9343 was cultured in a chemostat with glucose as growth limiting factor (*Dalland & Hofstad* 1974) We now report the effect of pH and dilution rate, and of different concentrations of glucose on the quantitative production of cell bound antigens extractable with 45 per cent aqueous phenol. This extraction method has previously been used for isolation of endotoxic lipopolysaccharide from *B. fragilis* (*Hofstad & Kristoffersen* 1970a)

MATERIALS AND METHODS

Continuous cultivation of *B. fragilis* NCTC 9343 and procedures for determination of the glucose concentration in the sterilized medium, cell yield and productivity have been described (*Dalland & Hofstad* 1974) The dilution rate D is the liquid flow rate divided by the total volume of medium in the fermentor

Cell-bound antigens were extracted by treating washed, acetone-dried whole cells with phenol/water for 15 min at room temperature (*Hofstad & Kristoffersen* 1970b) Following centrifugation at $2500 \times g$ for 30 min the water phase was pipetted off dialysed against running water and freeze dried The freeze-dried material, referred to as crude antigen, was used without further purification.

Antiserum against whole microbial cells was

TABLE 1 *Serological Activity of the Water Phase (Crude Antigen) from Phenol/Water Extractions of NCTC 9343 Grown in a Chemostat with Varying Amounts of Glucose in the Inflowing Medium, pH 7.0 Dilution Rate 0.07 h⁻¹*

Glucose in the medium per cent	Microbial cells mg dry wt/ml culture	Productivity mg dry wt/ml culture h	Crude antigen per cent of dry cells	Precipitating activity* µg/ml	Min. dose inhibiting HA§ µg
0.20	0 68	0 046	12.3	0.62	>5
0.38	1 06	0 074	11 7	0.31	5
0.55	1 46	0 102	11.5	0.31	2.5
0.69	1 64	0 115	12.4	0.62	1.25
0.87	1 76	0.123	14.9	0.62	5
1.05	1.66	0 116	13 7	1.25	5
1 40	1.63	0 114	18.2	0.62	5

* Least concentration (two-fold dilution) of antigen giving positive ring test.

§ Sensitizing antigen 25 µg/ml of a pool of crude antigen recovered from cells cultivated at pH 7.0, but at different dilution rates. 8 agglutinating units of antiserum.

TABLE 2. *Serological Activity of the Water Phase (Crude Antigen) from Phenol/Water Extractions of NCTC 9343 Grown in a Chemostat at Different Dilution Rates. pH 7.0*

Dilution rate h⁻¹	Glucose in the medium mg/ml	Microbial cells mg dry wt/ml culture	Productivity mg dry wt/ml culture h	Crude antigen per cent of dry cells	Precipitating activity* µg/ml	Min. dose inhibiting HA§ µg
0.05	6.5	1 45	0 073	9.9	0 62	1.25
0.07	6.5	1.57	0.110	8.7	0.62	1.25
0 11	5 6	1 17	0 128	11.8	0.62	0 16
0 14	5.8	0.67	0.094	11.0	0.31	0 62
0.16	6.2	0.32	0.051	13 4	0.31	0 62

* and § see legend Table 1

TABLE 3 *Serological Activity of the Water Phase (Crude Antigen) from Phenol/Water Extractions of NCTC 9343 Grown in a Chemostat at Different pH Dilution Rate 0.07 h*

pH	Glucose in the medium mg/ml	Microbial cells mg dry wt/ml culture	Productivity mg dry wt/ml culture h	Crude antigen per cent of dry cells	Precipitating activity µg/ml	Min. dose inhibiting HA µg
6.5	6.1	1 20	0 084	7 4	10.0	5.0
7 0	6.5	1.57	0 110	9.0	0.31	1.25
7.5	6..	0.65	0.046	15.6	2.5	5 0

and § see legend Table 1

produced in rabbits (*Hofstad* 1969) Methods for sensitization of sheep erythrocytes, for indirect haemagglutination (HA) and haemagglutination inhibition have been described (*Hofstad et al* 1971) Ring test precipitation was carried out in 2 mm wide tubes. Dilutions of crude antigen were layered over equal volumes of undiluted antiserum and precipitation recorded after incubation for 2 h at room temperature.

RESULTS AND DISCUSSION

The precipitating activity of the crude antigen did not seem to vary with the glucose concentration of the medium or with the dilution rate (Tables 1 and 2) A slight variation was observed with respect to the capacity to inhibit agglutination of sensitized sheep

erythrocytes. The variations were the same in repeated experiments. Cultivation at pH 6.5 and pH 7.5 was unfavourable not only for microbial yields and productivity but also with respect to serological activity of the crude antigen (Table 3). Of practical importance is that conditions optimal for microbial yield and productivity, *viz.* pH 7.0, 0.5–0.9 per cent glucose in the medium and dilution rates from 0.05 to 0.11 h^{-1} gave highly active antigen preparations.

The serological activity is comparable to that of phenol/water extracts of bacteria grown in batch culture without pH control (*Hofstad* unpublished work). However the yield of NCTC 9343 obtained in this laboratory by such cultivation has seldom exceeded 1 mg dry cells per ml of culture. Continuous cultivation of NCTC 9343 is thus a preferable cultivation method for production of microbial cells for immunochemical studies.

The precipitating activity and the capacity to inhibit haemagglutination of sensitized sheep cells did not parallell each other. This indicates that the two serological activities, at least partly, depend on separate molecular structures. Preliminary fractionation experiments support this suggestion.

REFERENCES

Dalland, E. & Hofstad, T.: Growth of *Bacteroides fragilis* NCTC 9343 in continuous culture and in batch cultures at controlled pH. Appl. Microbiol. In press, 1974.

Herbert, D., Elsworth, R. & Telling, R. C.: The continuous culture of bacteria; a theoretical and experimental study. J. gen. Microbiol. *14*, 601–622, 1956.

Hofstad, T.: Serological properties of lipopolysaccharide from oral strains of *Bacteroides melaninogenicus*. J. Bact. *97*, 1078–1082, 1969.

Hofstad, T. & Kristoffersen, T.: Chemical characteristics of endotoxin from *Bacteroides fragilis* NCTC 9343. J. gen. Microbiol. *61*, 15–19, 1970a.

Hofstad, T. & Kristoffersen, T.: Chemical composition of endotoxin from oral *Veillonella*. Acta path. microbiol. scand. Sect. B *78*, 760–764, 1970b.

Hofstad, T., Kristoffersen, T. & Maeland, J. A.: Serological properties of lipopolysaccharide from strains of oral *Veillonella*. Acta path. microbiol. scand. Sect. B, *79*, 615–619, 1971.

Pirt, S. J., Thackeray, E. J. & Harris-Smith, R.: The influence of environment on antigen production by *Pasteurella pestis* studied by means of continuous flow culture technique. J. gen. Microbiol. *25*, 119–130, 1961.

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PREPARATION OF TYPE SPECIFIC HERPES SIMPLEX ANTISERA BY AN IMMUNOSORBENT METHOD

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Jeansson, S. Preparation of type specific Herpes simplex antisera by an immunosorbent method. Acta path. microbiol. scand. Sect. B, 83 48–54 1975.

Herpes simplex virus (HSV) type 1 and type 2 specific immune sera have been produced by adsorption with glutaraldehyde-polymerized heterologous virus antigen. The adsorbed sera showed high specificity in complement fixation, immunoelectroosmophoresis and neutralization tests. *The precipitin patterns in IEOP indicate the presence of at least five HSV type 1 and three HSV type 2 specific antigens.*

Key words Herpes simplex antisera immunosorbent method.

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Specific antisera against Herpes simplex virus (HSV) type 1 and type 2 have been produced by cross absorption of rabbit immune sera with heterologous virus antigens (4 5 6, 11 12) Absorption was performed with either virus infected cells or crude extracts of infected cells. Such methods are hampered by nonspecific absorption of antibodies, incomplete absorption of heterologous antibodies and the formation of soluble antigen-antibody complexes. In order to achieve better absorption, HSV antigens were polymerized with glutaraldehyde before adsorption. Such an immunoadsorbent technique has previously been used in studies on serum-proteins (1) This technique gives an efficient adsorption without the production of soluble antigen-antibody complexes.

MATERIAL AND METHODS

Tissue culture BHK 21 clone 13(9) and GMK AH 1 cells were grown as previously described (6, 7) RK 13 *cells* ( ) *were* kindly supplied by *Tage Waller* Royal Veterinary College, Stockholm, and were grown in Eagle's MEM with 2 per cent of a 0.1 M l-glutamine and 10 per cent heated foetal bovine serum and without serum for maintenance. Before the change to maintenance medium, the tissue cultures were washed three times with phosphate buffered saline. All tissue culture media were supplemented with 100 units of penicillin *per ml and 100 µg of streptomycin per ml.*

Virus strains HSV type 1 strain Z originated from *D C Gajdusek* National Institute of Neurological Disease, Bethesda, Md. and HSV type 2 from *G Plummer* Loyola University of Chicago The passage history and origin of these strains have been described (6)

Preparation of antigens and immunoadsorbent For use in immunoelectroosmophoresis (IEOP) complement fixation (CF) and for preparation of

immunoadsorbent, viruses were grown in roller bottle cultures of BHK 21 cells as previously described (6) Antigens for IEOP and heat inactivated CF antigens were made as previously described (7)

Native antigens for CF were made by suspending 10 ml of packed HSV type 1 and HSV type 2 infected or noninfected BHK 21 cells in 20 ml of 0 025 M Tris-HCl buffer pH 8.0 Cell extracts were prepared by freeze-thawing (3 ×) treatment in a Potter-Elvehjelm homogenator at 2000 rev/min for 5 min at 0° C and ultracentrifugation (100000 × g for 60 min) The soluble antigen containing supernatant was concentrated by ultrafiltration on a Diaflo filter type PM 10 (Amicon Corp., Lexington, Mass.) to the starting volume of packed cells and was kept at –65 C. The pellet was also stored at –65 C and used in the preparation of immunoadsorbent.

The immunoadsorbent was prepared according to *Avrameas & Ternynck* (1) Two ml of pellet material was suspended in saline to give a final volume of 10 ml. One ml of 0.1 M acetate buffer pH 5.0 and three ml of 1 per cent glutaraldehyde (Schuchardt, München, Germany) solution were added and the suspension was stirred for 3 h at room temperature. After glutaraldehyde treatment the suspension was centrifuged for 15 min at 3000 × g and 4 C. The pellet was homogenized in 0.2 M phosphate buffer pH 7.2 and recentrifuged. The whole operation of homogenization and centrifugation was repeated three times.

Antisera HSV type 1 and type 2 grown in rabbit cells (RK 13) for 5 passages were kept at –65 C in 10 per cent dimethylsulfoxid (15) Infectivity titers were for HSV type 1 $10^{7.5}$ and for HSV type 2, 10^{6} $TCID_{50}$/ml *Ten rabbits*, weighing 2.5-3.0 kg and 6-8 months old, were immunized with HSV virus by infection of the shaved and scarified skin *bilaterally on the trunk* (5 rabbits with HSV type 1 and 5 with HSV type 2) (5) One month after the primary infection the rabbits were reinfected by infection of scarified cornea (0.05 ml homologous virus suspension) pretreated locally with Decadron® 0.1 per cent (8) Decadron® treatment was continued for one week. Almost all rabbits developed a herpetic keratitis. The rabbits were bled two weeks after healing of the corneal lesions. Sera were checked for titer and precipitating activity in IEOP and the most potent type 1 and type 2 sera were selected for adsorption.

Adsorption of antiserum Five ml of heated (56 C 30 min) rabbit immune serum was mixed with 1 ml packed heterologous immunoadsorbent. The mixture was stirred gently for 60 min at 37 C and overnight at 4 C and then centrifuged at 3000 × g for 15 min 4 C. The adsorption procedure was repeated twice. After adsorption, serum was centrifuged at 100000 × g for 60 min.

Serological tests CF tests were performed according to *Stadnyr et al.* (14) IEOP and plaque neutralization were performed as previously described (6, 7) In the neutralization test the plot of log per cent surviving virus against the quantity of antiserum used yielded a straight line. The relative potency of each serum is indicated by the numerical value of the slope (K value) of the

TABLE 1 *Chessboard Titration in Complement Fixation Test of the Herpes Simplex Type 1 Serum Pool before and after Adsorption with Heterologous Antigen*

..tn ..tigen	Antigen dilution	Unadsorbed HSV type 1 antiserum dilution							Adsorbed HSV type 1 antiserum dilution						
		4	8	16	3	64	128	256	4	8	16	32	64	128	256
SV type 1	200	+	+	+	+	+	–	–	+	+	+	+	–	–	–
antigen	400	+	+	+	+	+	–	–	+	+	+	+	–	–	–
	800	+	+	+	+	+	–	–	+	+	+	+	–	–	–
	1600	+	+	+	+	+	–	–	+	+	+	+	–	–	–
	3200	+	+	–	–	–	–	–	+	+	±	±	–	–	–
	6400	–		–	–	–	–	–	–	–	–	–	–	–	–
SV type 2	200	+	+	+	+	±	–	–	+	+	–	–	–	–	–
F antigen	400	+	+	+	+	±	–	–	+	±	–	–	–	–	–
	800	+	+	+	+	±	–	–	±	–	–	–	–	–	–
	1600	+	+	+	+	±	–	–	–	–	–	–	–	–	–
	3200	+	±	–	–	–	–	–	–	–	–	–	–	–	–
	6400	–	–	–	–	–	–	–	–	–	–	–	–	–	–

In chessboard titrations with uninfected control antigen tested against unadsorbed and adsorbed sera all reactions were *negative*

TABLE 2. *Chessboard Titration in Complement Fixation Test of the Herpes Simple Type 2 Serum Pool before and after Adsorption with Heterologous Antigen*

Native antigen	Antigen dilution	Unadsorbed HSV type 2 antiserum dilution 4	8	16	32	64	128	256	Adsorbed HSV type 2 antiserum dilution 4	8	16	32	64	128	256
HSV type 1 CF antigen	200	+	+	+	+	+	±	–	+	–	–	–	–	–	
	400	+	+	+	+	+	±	–	+	–	–	–	–	–	
	800	+	+	+	+	+	±	–	+	–	–	–	–	–	
	1600	+	+	+	+	+	±	–	–	–	–	–	–	–	
	3200	±	+	–	–	–	–	–	–	–	–	–	–	–	
	6400	–	–	–	–	–	–	–	–	–	–	–	–	–	
HSV type 2 CF antigen	200	+	+	+	+	+	±	–	+	+	+	+	–	–	
	400	+	+	+	+	+	±	–	+	+	+	+	–	–	
	800	+	+	+	+	+	±	–	+	+	+	+	–	–	
	1600	+	+	+	+	+	±	–	+	+	+	+	–	–	
	3200	+	+	+	+	+	–	–	+	+	+	±	–	–	
	6400	±	±	±	–	–	–	–	+	–	–	–	–	–	

In chessboard titrations with uninfected control antigen tested against unadsorbed and absorbed sera all reactions were negative.

straight line. The relative specificity of each serum is expressed as the ratio of the slopes of lines (10)

RESULTS

CF The results obtained in CF tests with native antigen of serumpools prior to and after adsorption with heterologous immunoadsorbent are given in Tables 1 and 2. Adsorption resulted in a considerable reduction (8-32 fold) of heterologous antibody activity while reducing the titer against homologous antigen by a factor of 2 to 4 Chessboard titrations in CF test with heat inactivated antigens gave similar results although antiserum titers were 2–4 times higher than with non heat inactivated antigens.

IEOP These serum pools were also tested in IEOP see Table 3 Prior to adsorption anti HSV 1 and 2 serum pools reacted both with homologous and heterologous antigens After adsorption precipitates were detectable with homologous antigens only The adsorbed HSV type 1 antiserum showed five precipitin lines with homologous antigen. The adsorbed HSV type 2 antiserum gave three precipitin lines with homologous antigen see Fig 1

VT The results of plaque neutralization tests are shown in Fig 2. The specificity of the antisera are expressed as the ratio of the slopes of lines. Before adsorption the ratio between K values for homologous and heterologous virus for anti HSV type 1 and 2 serum were 8.1 and 2.1 respectively After adsorption the ratio for anti HSV type 1 was 58.5 for anti HSV type 2 no neutralization of HSV type 1 virus could be detected and thus no ratio could be calculated.

DISCUSSION

With cross absorption methods using antigen extracts sera are diluted, contaminated with foreign proteins, contain heterologous virus antigen and highly anticomplementary probably due to the formation of soluble antigen-antibody complexes. The absorption procedure can only be repeated a few times because of a progressively increasing amount of foreign proteins in the absorption mixture, and because a significant nonspecific adsorption of specific antibodies may occur The converse is true with the glutaraldehyde polymerized antigen in each case The reduction of homologous CF activity (2-4 fold) observed after adsorption with heterologous

TABLE 3. *Chessboard Titrations in Immun oelectroosmopho esis of HSV Type 1 and Type 2 Antiserum Pools before and after Adsorption*

	Antigen dilution	Unadsorbed HSV type 1 antiserum dilution				Adsorbed HSV type 1 antiserum dilution				Unadsorbed HSV type 2 antiserum dilution				Adsorbed HSV type 2 antiserum dilution			
		1	2	4	8	1	2	4	8	1	2	4	8	1	2	4	8
HSV type 1 antigen	1	+ +‡	+ +	+	+	+ +	+	(+)	–	+ +	+ +	+	+	–	–	–	–
	4	+	+	+	+	+	+	(+)	–	+	+	(+)	(+)	–	–	–	–
	16	(+)	(+)	(+)	(+)	–	–	–	–	(+)	(+)	–	–	–	–	–	–
HSV type 2 antigen	1	+	+	+	+	–	–	–	–	+	+	(+)	(+)	+	–	–	–
	4	(+)	(+)	(+)	(+)	–	–	–	–	(+)	(+)	(+)	(+)	+	–	–	–
	16	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Dil tions are expressed as reciprocals.

‡ Symbols: – no precipitate, even after staining (+) very faint precipitate visible after staining + faint precipitate visible without staining + + strong precipitate visible without staining

In chessboard titrations with uninfected control antigen tested against unadsorbed and adsorbed sera all reactions were negative.

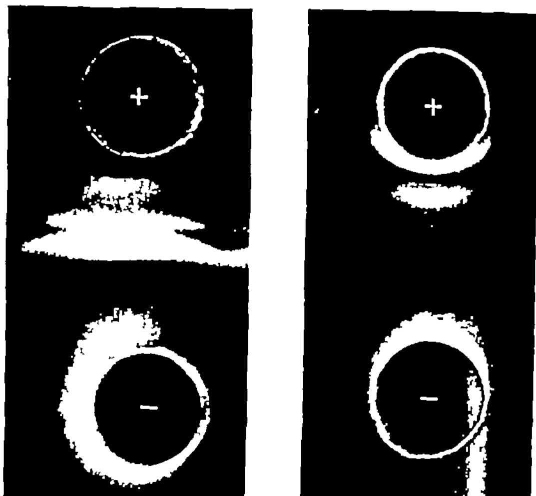


Fig 1 Precipitin pattern after immunoelectroosmophoresis of HSV type 1 and type 2 antigen against cross-adsorbed homologous sera. Both plates were stained with Coomassie brilliant blue.

Left + HSV type 1 cross-adsorbed antiserum.
— HSV type 1 antigen (native antigen) By examination of the original plate with a hand lens five precipitin lines were detected

Right + HSV type 2 cross-adsorbed antiserum.
— HSV type 2 antigen (native antigen).

virus antigen may be explained by the presence of high antibody activity to antigens common to types 1 and 2 or alternatively by nonspecific adsorption of homologous antibodies.

With glutaraldehyde polymerized HSV antigen it was possible to produce HSV antisera with high specificity in CF IEOP and neutralization tests. These antisera can be used in IEOP for typing of HSV strains. The precipitin patterns observed in IEOP indicate the presence of at least five HSV type 1 and three HSV type 2 specific antigens. Previous immunodiffusion studies (5 7 16) have demonstrated six HSV type 1 and two HSV type 2 specific antigens. The discrepancies between previous and present studies might be explained by the use of different immunization, absorption and immunodiffusion techniques.

Absorption methods to detect type specific antibodies have been developed (12 13) In one method heterologous or a separated type common, "Band II" antigen is used for ab-

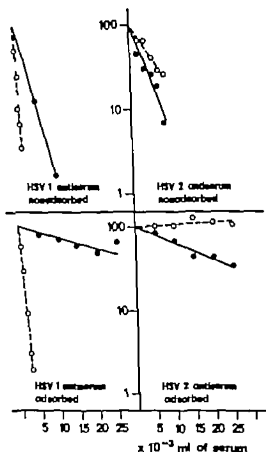


Fig 2 Multiplicity analysis of neutralizing potency of anti HSV type 1 and type 2 serumpools before and after adsorption with heterologous immunoadsorbent. The neutralization tests were performed as described under "Materials and Methods" Each point is an average for triplicate tissue culture tubes. The same virus preparations were used in all tests.

-o- -o- neutralization of HSV type 1
——•—•- neutralization of HSV type 2

sorption and type specific antibodies can be detected in neutralization tests.

In another method (13) the lysis of ⁵¹Cr labeled and HSV type 2 infected cells by Herpes type 2 antibodies in the presence of complement is used. The sera are rendered specific by prior absorption with non-labeled cells infected with HSV type 1 The test is sensitive but can only be used to detect antibodies against antigens present on the surface of the infected cell.

The use of glutaraldehyde polymerized antigens for adsorption of human sera make it possible to analyze human sera for the presence of type specific antibodies in conventional serological tests like CF as suggested by *Hildy* (17)

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REFERENCES

1 *Avrameas S & Ternynck, T.*, The crosslinking of proteins with glutaraldehyde and its use for the preparation of immunoadsorbents. Immunochemistry 6 53–66, 1969
2. *Bearle A J., Christofinos G C & Furminger I G S* Rabbit cells susceptible to rubella virus. Lancet 2 640–641 1963
3 *Ejercito P M Kieff E. D & Roizman B.*, Characterization of Herpes simplex virus strains differing in their effects on social behaviour of infected cells. J gen. Virol. 2 357–364 1968.
4 *Geder L. & Skinner G R. B.* Differentiation between type 1 and type 2 strains of Herpes simplex virus by an indirect immunofluorescent technique. J gen. Virol. 12: 179–182, 1971
5 *Honess R. W., Powell K. L., Robinson, D J Sim C & Watson D H*: Type specific and type common antigens in cells infected with Herpes simplex virus type 1 and on the surfaces of naked and enveloped particles of the virus. J gen. Virol. 22 159–169 1974
6. *Jeansson, S* Differentiation between Herpes simplex virus type 1 and type 2 strains by immunoelectroosmophoresis. Appl. Microbiol. 24 96–100 1972.
7 *Jeansson S.*, Production of Herpes simplex antisera with increased type specificity in guinea pigs by antibody suppression. In Press. Proc. Soc. Exp. Biol. Med.
8. *Kimura S J & Okumoto M* The effect of corticosteroids on experimental Herpes simplex keratoconjunctivitis in the rabbit. Am. J Ophthalmol. 43 131–134 1957
9 *MacPherson I A & Stoker M C. P* Polyoma transformation of hamster cell lones—an investigation of genetic factors affecting cell competence. Virology 16 147–151 1962.

10. *Savage T., Roizman B & Heine J W* Immunological specificity of the glycoproteins of Herpes simplex virus subtypes 1 and 2. J gen. Virol. *17*: 31–48, 1972.

11 *Schneweis K E & Nahmias A J.*, Antigens of Herpes simplex virus type 1 and 2—immunodiffusion and inhibition passive hemagglutination studies. Z. Immunitätsforsch. *141* 471–487 1971

12 *Sim C & Watson D H* The role of type specific and cross reacting structural antigens in the neutralization of Herpes simplex virus types 1 and 2. J gen. Virol. *19* 217–233 1973

13. *Smith J W Adam E. Melnick J L. & Rawls W E.* Use of the [51]Cr release test to demonstrate patterns of antibody response in humans to Herpesvirus types 1 and 2. J Immunol. *109* 554–564 1972.

14 *Svedmyr A Enders J F & Holloway A* Complement fixation with the three types of poliomyelitis viruses propagated in tissue culture. Am. J Hyg. *57* 60–70 1953.

15. *Wallis C & Melnick J L.* Stabilization of enveloped viruses by dimethyl sulfoxide. J. Virol. *2* 953–954 1968.

16. *Wildy P*: Herpesvirus and antigens. In Biggs, P M de The G. & Payne, L N (Ed.) Oncogenesis and Herpes viruses WHO International Agency for Research on Cancer Lyon. 1972, pp. 409–419.

17 *Wildy P* Antigens of Herpes simplex virus of oral and genital origin. Cancer Res. *33* 1465–1468, 1973

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APPLICATION OF ELECTROPHORESIS FOR SEPARATION OF TRIMETHOPRIM AND SULPHAMETHOXAZOLE IN COMBINED THERAPY

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Carlström, A., Dornbusch, K. & Hagelberg, A. Application of electrophoresis for separation of trimethoprim and sulphamethoxazole in combined therapy Acta path. microbiol. scand Sect. B, 83 55–60 1975.

Determination of the concentration of each drug in the combination trimethoprim: sulphamethoxazole was performed in patient sera by separation of the drugs by electrophoresis in agarose-gel followed by microbiological assay The electrophoretic method was equivalent to the methods used in the routine and showed good reproducibility

Key words Trimethoprim and sulphamethoxazole combined therapy separation electrophoresis.

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Microbiological assay for the determination of concentrations of antibiotics in body fluids is the most common method in general use (2, 3) In combined antibacterial therapy bacterial indicators which are sensitive to one of the antibacterial drugs to be determined can be used (14 15 16) When administered alone sulphonamides and trimethoprim can be assayed by microbiological methods (4 6) However the routine method for the determination of sulphonamides is based on chemical tests (5) and the determination of trimethoprim can be performed by spectrofluoreometry (17) Therefore serum samples from patients, administered with the combination of trimethoprim and sulphamethoxazole (Eusaprim® Bactrim® T+S®) are usually assayed by two separate determinations. Consequently it must be of great value to be able to determine simultaneously in a single operation the antibacterial activity of each component from a small volume of sample. This problem can be solved by *microbiological determination* subsequent to chemical inactivation of all drugs but one or subsequent to separation of the drugs by chromatography (11) or by electrophoresis (7 13)

In the present study electrophoresis in agarose-gel was used to separate trimethoprim and sulphamethoxazole used in combined therapy followed by microbiological assay of the separated drugs.

MATERIALS AND METHODS

Antibacterial drugs. Trimethoprim and sulphamethoxazole (Burroughs Wellcome Co., Beckenham, Kent, England) were dissolved in a minimum volume of 99.5 per cent ethanol and 1 M NaOH, respectively and then diluted in phosphate buffer pH 7.4–7.5 according to Dulbecco (9) to give a concentration of 1000 µg/ml and 10000 µg/ml, respectively. From these stock solutions, standard solutions were prepared in pooled human plasma. Each pool consisted of plasma from 4–6 healthy donors, pH 7.4–7.5. If necessary the pH of the pool was adjusted with 0.2 M KH_2PO_4 and each pool was tested for antibacterial activity. The ranges of the standard series were 1.0–16 µg/ml for trimethoprim and 20–320 µg/ml for sulphamethoxazole.

Electrophoresis. The electrophoresis equipment was made by AB Analysteknik, Vallentuna, Sweden. 25 ml of 0.8 per cent agarose (Miles-Seravac Ltd., Maidenhead, Berks, England) in 0.075 M barbital buffer pH 8.6, containing 2 mM calcium lactate and was spread on a glass-plate 200 × 100 × 1 mm. Wells, 3 mm in diameter were then punched in the agarose layer and 3 µl of standard solutions and patient sera were applied in each well by a micropipette. The electrode vessels were filled with barbital buffer. Bridges of filter paper connected the electrode vessels with the agarose layer. Electrophoresis was performed for 25 min at approximately 35 volt per cm.

Microbiological assay of trimethoprim and sulphamethoxazole after separation. Twenty-five millilitres of an antagonist-free agar (PDM-ASM-agar from AB Biodisk, Solna, Sweden) (8) containing 10^6–10^7 bacteria per ml of the indicator strain *Bacillus pumilus* (6) was poured over the agarose after electrophoresis. The seeded agar agarose plate was then incubated overnight at 37° C. Staining of the plates with Coomassie Brilliant Blue R 250 (Serva Fein, Biochemica, Heidelberg, Germany) was performed as described earlier (7).

Assay of trimethoprim by the disc diffusion method. The method according to *Bushby & Hitchings* (6) was used with the same antagonist-free medium and indicator strains as described above. The addition of 100 mg para-aminobenzoic acid per ml agar medium was used to inactivate sulphamethoxazole in the combination.

Standard curves. The diameters of inhibition zones caused by the known concentrations in the diffusion centres in the standard series were measured and plotted semilogarithmically (12). By simple reference to these standard curves the inhibition zones given by patient sera could be quantitated.

Assay of sulphamethoxazole by the chemical method. The method of *Bratton & Marshall* was used with sulphamethoxazole as a standard (5).

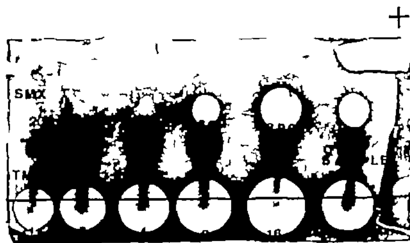


Fig. 1. Inhibition zones after separation of sulphamethoxazole (SMX) and trimethoprim (TMP) by electrophoresis in agarose-gel. Diluent: human plasma. The dark areas represent the plasma proteins after staining with Coomassie Brilliant Blue.
+ : anode —— line of application. — : cathode
Standard series: SMX 20–320 µg/ml.
TMP 1–16 µg/ml.

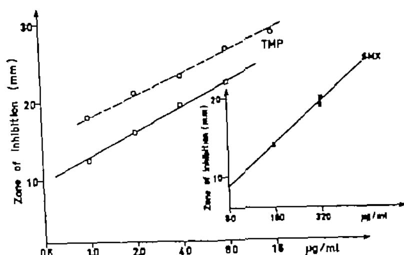


Fig 2 Standard curves for trimethoprim (TMP) determined by the electrophoretic method (– – –) and by the disc diffusion method (——) Inserted standard curve for sulphamethoxazole (SMX) determined by the electrophoretic method.

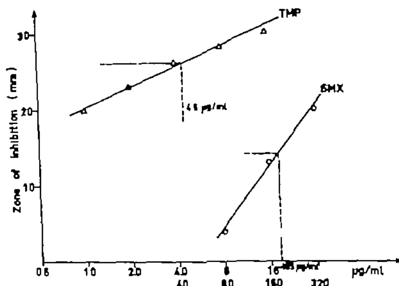


Fig 3 Standard curves for trimethoprim (TMP) and sulphamethoxazole (SMX) and patient sample determined by the electrophoretic method.

RESULTS AND DISCUSSION

As can be seen in Fig 1 separation of the components trimethoprim and sulphamethoxazole from a mixture by electrophoresis in agarose-gel was successful. Subsequent microbiological assay provided means of detecting and determining each component separately in small amounts of serum or plasma (5 µl) Sulphamethoxazole (single and in combination) like most of the serum proteins moved towards the anode, but trimethoprim remained at the site of application. Since the zones of inhibition were circular the concentrations of these drugs could be determined simply from standard semilogarithmic plots

TABLE 1 *Quantitation from Patient Samples of each Component in the Combination of Trimethoprim/Sulphamethoxazole by Different Methods*

Test sample	Concentration (μg/ml) of trimethoprim		Concentration (μg/ml) of sulphamethoxazole	
	Electrophoresis	Disc test‖	Electrophoresis	Chemical test
1	5.0*	4.8	75	72.0
2	5 0*	6.1	200*	151.5
3	12 0	5 7	135	125
4	6.4	4.9	90	98.5
5	2.5	3.2	61	66.2
6	6.2	5.2	–†	89 7
7	3.5	2 7	<80	38.8
8	1.0	1 6	<80	14 7

* The mean of two determinations.
‖ The mean of four determinations.
† The patient was on simultaneous therapy with azlocillin which zone of inhibition interferes with that of sulphamethoxazole.

TABLE 1 *Assays of Known Concentrations of Trimethoprim/Sulphamethoxazole in Mixture in Human Plasma by the Electrophoretic Method*

Trimethoprim Concentration expected (μg/ml)	Concentration obtained (μg/ml)	Sulphamethoxazole Concentration expected (μg/ml)	Concentration obtained (μg/ml)
1.5	1 4	30	<80
1.5	1.2	30	<80
3 0	2.9	60	<80
3.0	6.0	60	72
6.0	5 4	120	112
6.0	5 4	120	140
6 0	6.5	120	130
9 0	9.0	180	190
12	12	240	260
12	14	240	260

of concentration of antibacterial against diameter of inhibition zone (7) Samples with antibiotics highly bound to serum proteins, must be digested if circular inhibition zones are to be obtained (7) Fig 2 shows that the standard curves for trimethoprim were parallel by the electrophoretic method and by the disc diffusion method. The limit of the minimal measurable concentration was less than 0.5 μg per ml whether one or the other method was used.

Using sulphamethoxazole, straight lines were also obtained by the electrophoretic method (Fig 3) Using a not too heavy inoculum of bacteria and the thin agar layer the limit of the minimal measurable concentration was 40–80 μg/ml.

The electrophoretic method was applied to standard solutions of trimethoprim and sulphamethoxazole in mixtures together with serum samples from patients In addition, each standard solution and test sample was analysed by the disc diffusion method to determine trimethoprim and by the chemical method to determine sulphamethoxazole. Table 1 summarizes the results obtained from eight serum samples from patients on combined therapy The values determined by the elec

trophoretic procedure was comparable with those obtained by standard techniques. Using the electrophoretic method, a statistical analysis has been made and comparison with the analysis of the disc diffusion method described in an earlier paper (7) showed good agreement. The agar-diffusion bio-assay has also been reported to correlate well with chemical determinations (4 17) For further reassurance of the reproducibility of the electrophoretic method, five plasma samples with known concentrations of trimethoprim sulphamethoxazole in mixture were assayed one to three times. In each assay a standard series was used. Table 2 shows the good agreement between expected and obtained concentrations of each drug in the mixture.

The advantage of the electrophoretic separation combined with microbiological assay is that the antibacterial activity of each component in the mixture trimethoprim/sulphamethoxazole can be determined from a small volume in one step. Alternatively the concentration of trimethoprim in the mixture can be determined microbiologically after inactivation of sulphamethoxazole with PABA (6) and of sulphamethoxazole by means of a trimethoprim resistant strain. Although trimethoprim-resistant strains have been described in the literature (1) this latter method and the genetic stability of these strains has not been evaluated in our laboratories. Since these two drugs could be separated completely by electrophoresis, synergistic effects were avoided (10 18) Studies using the electrophoretic method are easy to perform the method does not require expensive equipment and the result is obtained within 16–20 hours.

REFERENCES

1 *Amyes S G B. & Smith J T.*, R-factor trimethoprim resistance mechanism: an insusceptible target site. Biochem. Biophys. Res. 58 412, 1974

2. *Bell, S M & Wood R.* An antibiotic assay method. J med. Lab. Technol 25: 27–32, 1968.

3 *Bennett J V., Brodie J L. Benner E. J &* *Kirby W M M* Simplified accurate method for antibiotic assay of clinical specimens. Appl Microbiol. 14 170–177 1966.

4 *Bergman H A., Baron, M & Weinstein L.* Serum sulphonamide concentration after oral administration Comparison of results of chemical assay with two bioassay techniques. Antimicrobial Ag. Chemother 1 68–273 1972.

5 *Bratton A. C. & Marshall Jr., E. K.:* New coupling component for sulfanilamide determination. J Biol. Chem. 128 537–550, 1939

6. *Bushby S., R. M & Hitchings G H.* Trimethoprim, a sulphonamide potentiator Br J Pharmacol. Chemother 33 72–90 1968.

7 *Carlström A., Dornbusch K., Hagelberg, A & Malmborg A.-S* Microbiological assay of mixtures of ampicillin and cloxacillin in plasma after separation by high voltage electrophoresis. Acta path. microbiol. scand. Sect. B, 82 67–74 1974

8. *Dornbusch K & Moore W B.* The effect of different media on the response of bacteria to sulphonamides and trimethoprim using the disc diffusion-method and regression line analysis. In "Trimethoprim/sulphapethoxazole in bacterial infections" pp. 39–51 (ed. Bernstein, L. S. & Salter A. J) Edinburgh, Livingstone, 1973

9. *Dulbecco R. & Vogt M* Plaque formation and isolation of pure lines with poliomyelitis viruses. J Exp. Med. 99 167–182, 1954

10 *Hitchings G H & Burchall J J* Inhibition of folatesynthesis and function as a basis for chemotherapy Adv Enzymol. 27 417–468, 1965

11 *Hochn M M., Murphy H W., Pugh, C T & Davis N E:* Paper chromatographic techniques for the determination of cephalothin and desacetylcephalothin in body fluids. Appl. Microbiol. 20 734–736 1970.

12. *Jalling, B., Malmborg, A.-S Lindman A &* Boréus, L. O: Evaluation of a micromethod for determination of antibiotic concentration in plasma. Europ. J Clin. Pharmacol. 4 150–157 1972.

13 *Lightbown J W & de Rossi P* The identification and assay of mixtures of antibiotics by electrophoresis in agar gel. Analyst. 90 89–98, 1965.

14 *Malmborg, A -S.:* Assay of antibiotics single or in combination, by agar diffusion technique. MD Thesis, 1974

15 *Naumann P., Lode H & Reinfjes E.* Zur Kombinationsbehandlung mit Penicillinen. Arzneim. Forsch. (Drug Res.) 23 218–223 1973.

16. *Sabath L. D., Loder P B., Gerstein, D A. & Finland M.* Measurements of three antibiotics (penicillin, cephalothin and chloram-

phenicol) when present together in mixtures. Appl. Microbiol. *16* 877–880 1968.

17 *Schwartz D E. Koechlin B. A & Weinfeld R. E.* Spectrofluorometric method for the determination of trimethoprim in body fluids. Chemotherapy *14* (Suppl.): 22–29 1969.

18. *Waterworth P M.* Practical aspects of testing sensitivity to trimethoprim and sulphonamide. Postgrad. med. J Nov Suppl. *45* 21, 1969

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BRIEF REPORTS

STUDIES ON THE PATHOGENICITY FOR RAT OF A MYCOPLASMA ISOLATED FROM RHEUMATOID ARTHRITIS

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Jansson, E., Reinius, S., Rimaila-Pärnänen, E. & Tuuri, S. Studies on the pathogenicity for rat of a mycoplasma isolated from rheumatoid arthritis. Acta path. microbiol. scand. Sect. B, 83: 61–62, 1975

The pathogenicity for rat of *M. arthritidis* strain Jasmin and strain 20-P from rheumatoid synovial tissue were studied. Only strain Jasmin produced signs of illness in four of the 12 rats infected. A mycoplasma was isolated from three tissue specimens. Ten of the 48 rats investigated showed mycoplasma antibodies. Five rats showed minor inflammatory changes in the distal joints of the limbs. The failure to induce arthritic symptoms in rats inoculated with strain 20-P from rheumatoid arthritis may be due to its 10-year long storage outside the animal host.

Key words: Mycoplasma; rheumatoid arthritis; pathogenicity; rat.

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In preliminary investigations performed in 1967–68 it was observed that a few isolates from rheumatoid synovial tissue when injected intraperitoneally killed mice weighing 9–12 g in 3–10 days (3). An earlier isolate 20-P from synovial tissue of a patient with definite rheumatoid arthritis was used in experiments on rats. It had been identified as related to *M. arthritidis*. A 50-fold concentrated suspension was injected intraperitoneally into rats weighing 120–200 g. The inoculum contained 10^{7} c.f.u./ml. After about three weeks, five of 12 rats injected developed signs of illness: loss of weight, difficulties in moving, limping, tenderness and swelling in the small joints of the lower extremities. One of them died. Antibodies against strain 20-P were found in the serum of a few of the animals one month later. The same lot of inoculum was also injected intracerebrally and 0.1 ml subcutaneously into 13 1½-day-old rats. Eleven of them died in 1–2 days. We therefore decided to study the rat pathogenicity of this strain 20-P in more detail and to compare it with the known rat pathogenic *M. arthritidis* strain Jasmin, which had been isolated by Klieneberger-Nobel from rat cancer tissue.

Material and Methods

Female rats weighing 150–170 g were used in four groups, each consisting of 12 animals. *M. arthritidis* strain Jasmin ATCC 14124 was received freeze-dried from the American Type Culture Collection. Strain 20-P had been passed several times on cell-free media and was stored at –20 C. 48-h actively growing cultures of both strains were titrated. Both showed > 10^{8} c.f.u./ml. Strain 20-P was injected in its 14th culture passage. The animals were inoculated into the tail vein, each rat receiving 0.5 ml/per 100 g body weight. Group A was given only mycoplasma broth medium, group B was inoculated with strain Jasmin, group C with strain 20-P and group D with the same strain concentrated 50-fold.

The amount of joint involvement was graded on an 0 to 5 basis as follows: erythema 1, swelling 1, fluid 1, difficulty in moving 1, paralysis 1, and symptoms in the small joints 0–1. Specimens of synovial tissue, spleen, liver and one kidney were taken when the animals were sacrificed. Every week, the rats showing signs of illness were killed and blood was taken by cardiac puncture. However, even if no symptoms of disease occurred,

some animals from each group were sacrificed every week. The experimental study was completed five weeks after inoculation. Mycoplasma isolation studies and antibody assays were performed as before (4). Histologically the distal joints of both upper and lower extremities from 46 rats were studied. The specimens were cut near the joints, fixed with formaldehyde and decalcified with EDTA solution according to *Balogh* (1) for 11–22 days.

Results and Discussion

In group B, inoculated with strain Jasmin, four of the 12 rats developed signs of illness. All the other three groups remained symptom-free. Three days after inoculation rats 5 and 6 in group B showed limping which was transient and gradually subsided. Six days after infection, rat 4 was limping with its left hind limb. The severity of the arthritis in the right tibiotarsal joint was scored 3, in the left 1, and in the small joints of both upper extremities 1. The animal had also lost weight and was therefore sacrificed one week after inoculation. Rat 9 on day 4 showed severe conjunctivitis.

TABLE 1. *Mycoplasma Antibodies in Rats Measured by the Indirect Hemagglutination Test*

Rats group	No.	Weeks after inoculation	Antibody titre against 20-P	Antibody titre against Jasmin
A	2	1	32	128
A	7	5	8	4096
B	5	2	2048	4096
B	6	3	256	<8
B	9	5	128	128
B	10	5	64	<8
B	12	5	<8	512
D	3	1	128	128
D	7	5	16	16
D	8	5	64	256

A mycoplasma was isolated in three cases from synovial tissue of rat 4 in group B and from the liver and spleen of one rat in groups C and D. The latter specimens were taken one and two weeks after inoculation. All the other 142 specimens taken were negative for mycoplasma. Ten of the 48 rats investigated showed mycoplasma antibodies (Table 1). Two animals in control group A probably contracted an infection. They were kept in the same room with the others. The highest antibody titres were observed two weeks after inoculation.

Histologically the most distinct inflammatory changes were found in group B, rat 4, in the radiocarpal joint one week after infection. There was perivascular cuffing of mononuclear cells, most of them lymphocytes but also a few neutrophils. In group C, rat 1 showed after one week in the periarticular tissue of the first metatarsophalangeal joint a very slight inflammatory reaction. Rat 6 in the same group revealed after three weeks in the corresponding joint slight lymphocyte infiltration beneath the synovial membrane. At five weeks after inoculation a rat in group D showed cuffing of mononuclear cells in the periarticular tissue of the tibiotarsal joint. Another rat in the same group revealed in the proximal periostal tissue of the corresponding joint a local perivascular inflammatory reaction. The liver tissue specimens of several rats showed slight infiltration of mononuclear cells.

The failure to induce arthritic symptoms in rats inoculated with strain 20-P from rheumatoid arthritis may be due to its 10-year storage outside the animal host. The preliminary investigations of its pathogenicity were performed three years after its primary isolation. Other scientists have reported that cultivation on cell-free media often results in a loss of pathogenicity (2, 5). This opinion gains further support from the fact that in our study the known rat pathogen *M. arthritidis* strain Jasmin produced illness in only four out of 12 rats. In future studies, therefore, only newly recovered strains should be used. These might be valuable because, as *Thomas* stressed, an animal model provides the possibility of examining several aspects of mycoplasma disease (6).

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References: 1 *Balogh K.*, J Histochem. Cytochem. *10* 232, 1961. — 2 *Barden J. A. & Tully J. G.* J. Bact. *100* 5–10 1969. — 3 *Jansson E., Vainio U., Snellman O. & Tuuri S.* Ann. rheum. Dis. *30* 413–418, 1971. — 4 *Jansson E., Mäkisara P., Vainio K., Snellman O. & Tuuri S.*: Acta Rheum. Scand. *17* 227–235 1971. — 5 *Schütze E.* Zbl. Bakt., Abt. I (Orig.) *208* 301–329 1968. — 6 *Thomas L.*, Federation Proc. *32* 143–145, 1973.

OCCURRENCE OF K88-NEGATIVE *ESCHERICHIA COLI* SEROTYPES IN PIGS WITH POST WEANING DIARRHOEA

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Riising, H. J., Svendsen, J. & Larsen, J. L. Occurrence of K88-negative *Escherichia coli* serotypes in pigs with post weaning diarrhoea. Acta path. microbiol. scand. Sect. B, 83 63–64 1975.

Faecal swabs from pigs with post weaning *E. coli* diarrhoea in 43 different herds were bacteriologically examined. A survey of the isolated haemolytic *E. coli* strains showed that only 1 of the examined strains possessed the K88 antigen and that *E. coli* strains belonging to serogroup O149:K91 were most frequently isolated. The results suggest that the K88 antigen is of minor significance in the development of post weaning diarrhoea associated with *E. coli* serotype O149 K91

Key words *E. coli* serotypes pigs K88 antigen enteric diseases.

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One of the characteristics of porcine enteropathogenic *E. coli* organisms is their ability to adhere to and to colonize the upper intestinal tract (*Smith & Halls* 1968, *Arbuckle* 1970) Most of the enteropathogenic *E. coli* organisms isolated from cases of neonatal colibacillosis contain the K88 antigen which confers adhesive properties to the bacteria (*Stirm et al* 1967) and it has been suggested (*Smith & Linggood* 1971 *Jones & Rutter* 1972) that this plasmid-determinated antigen was responsible for the establishment and multiplication of K88-positive bacteria in the small intestinal tract of pigs.

During outbreaks of post weaning *E. coli* diarrhoea in few Danish herds none of the isolated and typed *E. coli* strains possessed the K88 antigen (*Svendsen et al* 1974) although O-group 149 was most frequently present. In order to further study the consistency of this observation, faecal swabs from 2 3 acute cases of post weaning diarrhoea were collected in 42 herds from different parts of the country A total of 88 samples were examined. Furthermore all fatal cases of weaning diarrhoea from an additional herd (herd D cf. *Nielsen et al.* 1974) were subjected to *post mortem* and bacteriological examination, and faecal swabs from scouring litter mates were examined.

In cases where the bacteriological examination revealed predominance of haemolytic *E. coli* several colonies were selected from each plate for typing, using a rapid slide agglutination test with OK-antisera, as described by *Sojka* (1965) Selected *E. coli* strains were re-typed at the *International Escherichia coli Centre* Statens Seruminstitut, Copenhagen.*

The results of the examinations are presented in Table 1 It is apparent that *E. coli* strains belonging to serogroup O149 K91 were most frequently isolated and that only one of all the typed strains possessed the K88 antigen. These observations are contrary to the findings on *E. coli* isolated from

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TABLE 1 *Survey of Isolated Haemolytic* E. coli *Serotypes from Acute Cases of* Post *Weaning Diarrhoea*

	Samples from 42 herds	Samples from herd D	
		Dead pigs	Faecal swabs
No. of samples	88	37	19
Serotypes dominating the intestinal flora			
O149 K91 K88ab	1	0	0
O149.K91	35	31	10
O147 —	8	0	0
O141.K85ab	5	0	0
O139.K82	2	0	0
O138.K81	1	0	4
O8 .K87	1	0	0
Others or non-typable	13	3	0
Intestinal flora without haemolytic *E. coli*	24	3	5
Total	88	37	19

neonatal *E. coli* diarrhoea, where most of the typed strains possess the K88 antigen (*Sørendsen et al.* 1975)

The results indicate that the K88 antigen is of minor significance in the development of *post* weaning diarrhoea associated with *E. coli* serotype O149.K91 If adhesion of enteropathogenic *E. coli* strains is a prerequisite for development of enteric *E. coli* infections, factor(s) other than the K88 antigen must be responsible for such adhesion in weaned pigs.

References 1 *Arbuckle J B. R* J Med. Microbiol. *3* 333–340 1970.—2. *Jones G W & Rutter J M* Infect. Immunity *6* 918–927 1972.—3. *Nielsen N C. Christensen K., Bille N & Larsen J L.* Nord. Vet.-Med. 26 137–150, 1974 —4 *Smith H W & Halls S* J Med. Microbiol. *1* 45–49 1968.—5 *Smith H W & Linggood, M A* J Med. Microbiol. *4* 467–485, 1971.—6. *Sojka W J* *Escherichia coli* in domestic animals and poultry Commonwealth Agricultural Bureau, Farnham Royal 1965 pp. 193–214.—7 *Stirm S., Orskov F., Orskov I & Birch-Andersen A* J Bacteriol. 93 740–748, 1967.—8. *Sørendsen, J., Larsen J L. & Bille N* Nord. Vet. Med 26 314–322. 1974.—9. *Sørendsen J., Bille N Nielsen N C Larsen J L. & Riising H J* Nord. Vet. Med. 27 1975 in press.

DIFFERENTIATION BETWEEN *PSEUDOMONAS CEPACIA* AND *PSEUDOMONAS PSEUDOMALLEI* IN CLINICAL BACTERIOLOGY

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Bremmelgaard, A. Differentiation between *Pseudomonas cepacia* and *Pseudomonas pseudomallei in clinical bacteriology* Acta path. microbiol. scand. Sect. B 83 65–70 1975

If only conventional laboratory tests are used, the widely occurring species *Pseudomonas cepacia* may due to its variability sometimes be difficult to differentiate from the dangerous pathogen *Pseudomonas pseudomallei*. In a comparative study using fresh isolates it is shown that animal inoculation seems to be a useful additional method in the differentiation. Also the determination of the ability to utilize selected carbon sources such as starch, hydroxybenzoate and uracil is helpful in reaching a correct identification.

Key words *Pseudomonas cepacia* *Pseudomonas pseudomallei* differentiation.

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Burkholder (3) described *Pseudomonas cepacia* as a plant pathogen, but later reports (2 5 6 10 13 18 21) have shown that the organism can also be pathogenic for man. Generally the virulence seems to be low although it is clear that to a severely debilitated patient infection with *P cepacia* may constitute a serious additional risk. The report of a subacute necrotizing granulomatous lung lesion resembling melioidosis in a previously healthy young man (5) indicates that strains with greater virulence may possibly occur

Systematically *P cepacia* together with *P pseudomallei* *P mallei* *P marginata* and *P caryophylli* constitute as shown by *Ballard et al* (1) a DNA homology subgroup within the aerobic pseudomonads with a number of cultural and metabolic characters in common.

As *P pseudomallei* is a human pathogen usually causing serious infections, its differentiation from the related, but far less dangerous *P cepacia* has become an issue of some importance for the medical bacteriologist. According to current views (14 19) it is true that *P pseudomallei* is restricted to a limited geographical area in the tropics and would therefore not be expected to appear outside this area. However it is so unusual for bacteria to have a restricted geographical distribution that one would not be surprised if a closer study revealed the occurrence of *P pseudomallei* also in other parts of the world or cases of melioidosis without any direct connection with the endemic area.

The incident which gave rise to the present

TABLE 1 *Bacterial Strains*

Species	Designation	Origin
P cepacia	AB 1164 King 7179	Unknown
P cepacia	AB 1424* ATCC 17559	Forest soil
P cepacia	AB 1425* ATCC 17560	Forest soil
P cepacia	961/67*	Urine
P cepacia	D 142*	Ascitic fluid
P cepacia atypical	AB 1934*	Blood
P pseudomallei	AB 1936* NCTC 4845	Laboratory monkey
P pseudomallei	AB 1937* NCTC 10274	Blood
P pseudomallei	AB 1938* NCTC 10276	Human
P pseudomallei, atypical	PJ 54	Unknown

* Numbers from the strain collection at the Department of Diagnostic Bacteriology Statens Seruminstitut Copenhagen.
The three NCTC-strains were received from the National Collection of Type Cultures, London, and originated from natural cases of melioidosis.

report was the observation in a fatal case of bacteriaemia in a 4-year-old boy suffering from repeated septic infections that a strain of *Pseudomonas* was isolated which, according to the conventional tests, *could not* be definitely identified as either *P cepacia* or *P pseudomallei* Additional tests including animal inoculations proved it to be an atypical strain of *P cepacia*

MATERIALS AND METHODS

Bacterial strains are shown in Table 1

Animals Male guinea pigs, albino, international designation Ssc.Al 2 months old weighing about 600 g.

Methods

Bacteriological routine tests essentially as described by *Jessen* (9) except that indol test, reduction of nitrate and oxidation/fermentation tests were made at 35 and 30 C and not at 23 C.

The decarboxylase tests according to *Møller* (12)

The ONPG test according to *Bülow* (4)

The phenylalaninedeaminase test was made by preparing a heavy suspension of bacteria from an agar plate in 1 ml of a watery non-buffered solution of 0.2 per cent L-phenylalanine $FeCl_3$ was added after about 20 hours and a very dark green colour indicated that the test was positive

Growth in mineral media was carried out in Hutner's mineral base (17) with 0 1 per cent $(NH_4)_2SO_4$ as N source and 0 1 per cent acetic acid, butyric acid, starch, o-hydroxybenzoate, m-hydroxybenzoate and uracil as C sources. Three transfers in fluid medium were performed before final reading. Transfer was made when growth was visible. When no turbidity was observed after 8 days of incubation, it was concluded that the strain was unable to utilize the carbon source.

As testosterone is insoluble and reading for growth therefore is difficult in a fluid medium, growth experiments on testosterone were made on agar plates containing Hutner's mineral base with 0 2 per cent testosterone. One drop of a suspension containing 10^3 bacteria per ml in Hutner's mineral base was spread on a nutrient agar plate, an agar plate containing Hutner's mineral base with testosterone and a similar plate without testosterone The plates were incubated at 30° C and reading was made after 2, 4 and 6 days.

Methods for animal inoculation The strains were grown in 200 ml flasks with about 20 ml filtered broth medium containing 5 per cent serum, 0.01 per cent glucose and 0 05 per cent haemoglobin, and incubated overnight at 35 C.

Male guinea pigs weighing about 600 g were inoculated intraperitoneally with culture containing about 10^8 microorganisms per ml. Two animal received 0 1 and 0.5 ml respectively of each strain. Animals which died were autopsied as soon after death as possible The remaining animals were killed with carbondioxide after 4 days and then autopsied. For bacteriological examination samples were in all cases taken from peritoneum, liver spleen, lymph nodes in the mesenterium, heart blood and lungs. Samples from testes were taken from most of the animals. The samples were seeded on blood agar plates and incubated at 35 C for 2 days. Colonies appearing on the plates were subsequently identified.

RESULTS

ll strains were medium-sized, motile, gram-negative rods. They grew well on agar plates at 30 C. The five typical *P cepacia* produced smooth colonies with circular periphery AB 1934 also produced such colonies but in addition others with irregular periphery The three typical *P pseudomallei* grew with rough colonies showing a metallic sheen. PJ 54 also produced rough colonies but without the characteristic sheen. In broth, all strains produced a surface pellicle. The pellicle formed by the four *P pseudomallei* showed, in contrast to that formed by the other strains, a strange oily sheen. Three *P cepacia* were pigmented on tubed semi solid nutrient agar AB 1164 and AB 1424 produced a yellow and AB 1425 a violet pigment.

The following tests were positive in all strains oxidase reaction, acid production after 1–3 days from arabinose, xylose glucose, galactose, maltose dulcitol, sorbitol mannitol, inositol and glycerol and growth with acetic and butyric acid. In the latter medium all strains produced inclusions. Flagella staining showed them to be polar multitrichous.

The following tests were negative in all strains urease test, phenylalaninedeaminase test, indol production, H_2S production, acid formation from rhamnose, melezitose and inulin.

Reactions in which different strains gave different results are shown in Table 2.

Reactions which completely separate the five typical *P cepacia* and the three typical *P pseudomallei* are shown in Table 3

Animal Inoculation

None of the 10 animals injected with the five typical *P cepacia* nor the two animals inoculated with the atypical *P pseudomallei* PJ 54 showed any sign of illness during the 4 days of observation. Post mortem, there were no visible changes in the organs of three animals. Nine animals showed slight swelling of the lymh nodes in the mesenterium. Seeding from liver spleen and lymph nodes from the animal injected with 0.1 ml of AB 1164 showed growth of a few colonies of the injected strain. The organs from the other animals were sterile.

The two animals inoculated with the atypical *P cepacia* AB 1934 were also killed after 4 days. The one which was injected with 0 1 ml showed no changes in the organs and no organisms were recovered. The other animal inoculated with 0.5 ml of the strain was grossly dehydrated. There were fibrinous peritonitis, bleeding in the lymph nodes in the mesenterium, fat degeneration of the liver and swelling of the spleen. From all organs including the testicles a great number of colonies were grown.

The six animals injected with the three atypical *P pseudomallei* strains died after 1–2 days of uncharacteristic illness. Examination of the organs showed a rather uniform picture, *viz.* intense fibrinous peritonitis, swelling and bleeding of the lymph nodes in the mesenterium, many necrotic and purulent lesions in the liver spleen, kidneys, gut and diaphragma. The testicles were slightly swollen and the lungs were condensed and hyperaemic. In some animals the suprarenal glands were hyperaemic. Seeding from the organs showed growth of a great number of colonies.

DISCUSSION

The characters of the isolate AB 1934 that made its identification as *P cepacia* difficult were above all its ability to grow at 42 C and its lack of a lysine decarboxylase because these properties are typical of *P pseudomallei* and considered of special significance in the differentiation of the two species (8, 11 14 15 16 21) Furthermore, the results of the nitrate reduction test and the ornithin decarboxylase test, positive and negative, respectively—although compatible with both diagnoses—on statistical grounds rather suggested *P pseudomallei* than *P cepacia*. Finally AB 1934 could not liquefy gelatine, a property possessed by both *P pseudomallei* and *P cepacia* thus this result also complicated the identification.

TABLE 2. *Reactions in which Di[...]*

	5 typical *P cepacia*	3 typical *P pseudomallei*	Atypical *P cepacia* AB 1934	Atypical *P pseud* PJ 54
Sucrose	4+ 1—	3+	—	+
Lactose	2+ 3×	3+	+	+
Fructose	5×	3+	+	+
Trehalose	4+ 1×	3+	+	×
Melibiose	1× 4—	3—	—	—
Raffinose	3× 2—	3+	—	—
Starch	5—	3+	—	+
Salicin	5—	3+	—	—
Ethanol	2× 3—	3+	—	—
Erythritol	5—	3×	—	×
Adonitol	5×	2+ 1×	+	—

\+ = Acid production on day 1–3.
× = Acid production on day 4–28
— = No acid production on day 28.

TABLE 3 *Reactions which Completely Separate the Five Typical* P cepacia *and the Three Typical* P pseudomallei

	Five typical *P cepacia*	Three typical *P pseudomallei*	Atypical *P cepacia* AB 1934	Atypical *P pseudomallei* PJ 54
Acid production from starch	—	+	—	+
Acid production from salicin	—	+	—	—
Acid production from erythritol	—	×	—	×
Growth at 42° C	—	+	+	—
Lysine decarboxylase	+	—	—	—
Arginine dihydrolase	—	+	—	+
Growth on starch	—	+	—	+
Growth on o-hydroxybenzoate	+	—	+	—
Growth on m-hydroxybenzoate	+	—	+	—
Growth on uracil	+	—	+	—
Animal inoculation resulting in death	—	+	—	—

A special difficulty in this case was that the *P pseudomallei* strain PJ 54 which was first used in the comparison, turned out to be a very defective one with the consequence that several test results, such as gelatine liquefaction, acid production from raffinose, salicin and ethanol, at first appeared to be compatible with the diagnosis *P pseudomallei*.

Not until three typical strains of *P pseudomallei* were included in the comparison, and not until growth experiments with selected carbon and energy sources and animal inoculations had been added to the conventional tests did the true facts of the case transpire. According to these facts—shown in Table 3—it is beyond doubt that strain AB 1934 must be identified as a strain of *P cepacia* although the strain is atypical in growing at 42 C, in lacking a lysine decarboxylase and in being unable to liquefy gelatine.

[T]able ... Gave Different Results

	5 typical *P cepacia*	5 typical *P pseudomallei*	Atypical *P cepacia* AB 1934	Atypical *P pseudomallei* PJ 54
[...]on of nitrate to nitrite	2+ 3—	5+	+	+
[...]urther reduced	5—	2+ 1—	—	—
[...] at 42° C	5—	5+	+	—
[...] liquefaction	5+	5+	—	—
	2+ 3—	5—	+	—
[...]e dihydrolase	5—	5+	—	+
[...] decarboxylase	5+	5—	—	—
[...]ne decarboxylase	5+ 2—	5—	—	—
[...]i on starch	5—	5+	—	+
[...]i on o-hydroxybenzoate	5+	5—	+	—
[...]i on m-hydroxybenzoate	5+	5—	+	—
[...]i on uracil	5+	5—	+	—
[...]h on testosterone	5+ 2—	5—	—	—

Gelatin liquefaction tubes were read for the last time on day 14

The test results listed in Table 3 demon[s]trate that a differentiation between the two [s]pecies, *P cepacia* and *P pseudomallei*, can [u]sually be obtained by the aid of conventio[n]al tests, *i.e.* the six first in the table. If, in the [c]ase of atypical strains, additional tests are [d]eemed necessary both the examination for [u]tilizable carbon sources and animal inocula[t]ions are useful. Since most microbiological routine laboratories will probably consider the determination of utilizable carbon sources rather unfamiliar animal inoculations are likely to be the preferred additional test.

It has been shown that a characteristic testicular lesion, the Straus reaction, develops in male guinea pigs after intraperitoneal inocula[t]ion of *P pseudomallei* (7 20). It consists in swelling of the testicles in 2 to 3 days by the [1]0th day they are greatly enlarged and [u]lceration may occur However if the an[im]als are inoculated with a greater amount [o]f culture, they will die before the Straus [r]eaction develops. This happened to the six [a]nimals inoculated with the three typical *P* [p]*seudomallei*. They died after 1 to 2 days [a]nd showed only a slight swelling of the [t]esticles, which might have been overlooked [h]ad it not been especially looked for

On the other hand, *Bassett et al.* (2) reported some swelling of the testicles in a guinea pig 7 days after intraperitoneal inoculation of *P cepacia*. If animal inoculation is used it therefore seems important to make death the deciding criterion, also because macroscopically visible pathological changes and positive cultures from some internal organs were observed in some of the animals that had received injections of *P cepacia*. Although it was in all cases obvious from the direct comparisons that these latter changes were far less pronounced in animals receiving *P cepacia* than in those receiving *P pseudomallei* the atypical *P cepacia* strain AB 1934 did produce fairly marked changes. Owing to the limited number of strains and animals used in this study it is not possible to state categorically that animal inoculation can always be relied on in the differentiation but the outcome is sufficiently suggestive to recommend the method if the results of the conventional tests indicate the need for additional evidence.

REFERENCES

1 *Ballard, R. W., Palleroni, N J Doudoroff M., Stanier R. Y & Mandel M* Taxonomy of the aerobic pseudomonads *Pseudomonas cepacia, P marginata, P alliicola* and *P caryophylli*. J gen. Microbiol. 60 199–214 1970.

2. *Bassett D C J., Stokes K J & Thomas W*

R G Wound infection with *Pseudomonas multivorans*. A water-borne contaminant of disinfectant solutions. Lancet *1* 1188–1191 1970
3 *Burkholder W H.*, Sour skin, a bacterial rot of onion bulbs. Phytopathology *40* 115–117 1950.
4 *Bülow P.*, The ONPG test in diagnostic bacteriology Acta path. microbiol. scand. *60* 376–386 1964
5. *Dailey R. H & Benner E. J.*, Necrotizing pneumonitis due to the pseudomonad "Eugonic Oxidizer-Group I" New Engl J Med. *279* 361–362, 1968.
6 *Ederer G M & Matsen J M* Colonization and infection with *Pseudomonas cepacia*. J infect. Dis. *125* 613–618, 1972.
7 *Garry M W & Koch, M L.* Chronic melioidosis Bacteriologic and clinical correlation in diagnosis. J Lab. clin. Med. *38* 374–383 1951
8. *Gilardi G L.* Characterization of *Pseudomonas* species isolated from clinical specimens. Appl. Microbiol. *21* 414–419 1971
9 *Jessen O Pseudomonas aeruginosa* and other green fluorescent pseudomonads. A taxonomic study Munksgaard, Copenhagen 1965, pp. 38–52.
10 *Jonsson V* Proposal of a new species *Pseudomonas kingii* Int. J system. Bact. *20* 255–257 1970
11 *Lapage S P., Hill L. R & Reeve J D Pseudomonas stutzeri* in pathological material. J med. Microbiol. *1* 195–202 1968.
12 *Møller V* Simplified tests for some amino acid decarboxylases and for the arginine dihydrolase system. Acta path. microbiol. scand. *36* 158–172 1955
13. *Phillips, I., Eykyn S., Curtis M A. & Snell, J J S Pseudomonas cepacia* (multivorans) septicaemia in an intensive-care unit. Lancet *1* 375–377 1971
14 *Redfearn M S Palleroni N J & Stanier R. Y.*, A comparative study of *Pseudomonas pseudomallei* and *Bacillus mallei*. J gen. Microbiol *43* 293–313 1966.
15. *Salisbury W A & Likos J J Pseudomonas pseudomallei* in a case of chronic melioidosis. Amer J clin. Path. *54* 602–606, 1970.
16. *Snell J J S., Hill, L. R., Lapage S P & Curtis M A*: Identification of *Pseudomonas cepacia* Burkholder and its synonymy with *Pseudomonas kingii* Jonsson. Int. J system. Bact. *22* 127–138 1972.
17 *Stanier R. Y., Palleroni, N J & Doudoroff, M.*, The aerobic pseudomonads a taxonomic study J gen. Microbiol. 43 159–271 1966
18. *Taplin D Bassett D C. J & Mertz, P M.* Foot lesions associated with *Pseudomonas cepacia*. Lancet 2 568–571 1971.
19 *Thin R N T., Groves M Rapmund, G. & Mariappan M Pseudomonas pseudomallei* in the surface water of Singapore. Singapore med. J *12* 181–182 1971
20 *Wilson G S & Miles A A* Topley and Wilson's principles of bacteriology and immunity 5th Edition, vol. *1* Edward Arnold (Publishers) Ltd. London 1964 pp. 626–633
21 *Yabuuchi E. Miyajima, N., Hotta, H. Ohyama, A & Tanaka H Pseudomonas cepacia* from blood of a burn patient. Med. J Osaka. Univ *21* 1–6 1970.

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STUDIES ON THE GROWTH OF FELINE PANLEUKOPAENIA VIRUS IN CELL CULTURES

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The growth of feline panleukopaenia virus was examined in relation to various cell growth curves of secondary feline kidney cells. In cell cultures with a high mitotic index, the manifestation of virus infection, expressed as percentage number of cells with inclusion bodies, was correspondingly high. In cultures with low mitotic index, the percentage number of cells with inclusion bodies was low. A cytopathic effect was seen by inoculation of virus with a titre of $10^{4.7}TCID_{50}/ml$. This effect was most pronounced using cell cultures seeded in a quantity of 1 million cells per ml to 500,000 cells per ml with inoculation made during the first 24 hours after seeding when the mitotic index was high. Both the cytopathic effect and the occurrence of inclusion bodies were of transient nature. The peak of infection, both with regard to the cytopathic effect and the percentage number of cells with inclusion bodies, was seen three days after inoculation.

Key words: Feline panleukopaenia virus; growth; cell cultures.

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A previous report (*Flagstad* 1973) described the isolation of feline panleukopaenia virus in feline kidney cell cultures. It was observed that growth of the virus seemed to depend on high mitotic activity of the cell cultures. A cytopathic effect occurred occasionally in the form of roughening and thinning of the cell sheet with scattered opaque dead nuclei in random patches. The virus produced intranuclear inclusion bodies which were detectable in stained preparations. The cytopathic effect occurred irregularly in the cultures and appeared to be transient. This also characterized the intranuclear inclusion bodies.

The aim of the present work was to study the growth of the virus in relation to the mitotic activity in cell cultures, and to examine the conditions under which the cytopathic effect and inclusion bodies occurred.

MATERIAL AND METHODS

Cell cultures. Primary monolayer cultures of kitten kidney cells were prepared according to the method of *Madin et al.* (1957). The cells were kept as bottle cultures at 37° C until a confluent layer of cells was obtained. Growth of the cells in the

primary cultures was not uniform and therefore secondary cell cultures in which uniform growth was obtained were used.

For making secondary cell cultures, the cell sheet was covered with trypsin-versene solution. The trypsin-versene solution was removed after 30 seconds and the bottles incubated at 37 C until the cell sheet began to slough. The cells were resuspended in growth medium to contain 2 million cells per ml, 1 million cells per ml and 500,000 cells per ml. Leighton tubes were seeded with 2 ml of cell suspension and incubated at 37 C.

The medium used for primary cell cultures was Hanks' balanced salt solution (Hanks BBS) to which was added 0.05 per cent lactalbumin hydrolysate, 0.01 per cent yeast extract and 10 per cent calf serum. G-penicillin 100 IU/ml, dihydrostreptomycin 100 mg/ml and mycostatin 100 IU/ml were added. The same medium with the 10 per cent calf serum substituted by 10 per cent foetal bovine serum was used for secondary cell cultures. The medium in the Leighton tubes was renewed every third day

Cell count Direct determination of the number of cells by means of a haemocytometer was made when the cell suspension was seeded in Leighton tubes, at the time of inoculation, and at the end of the incubation period. For determination at time of inoculation and the end of the incubation period, the cell sheet was washed with buffered phosphate and the cells brought in suspension by trypsin-versene solution. The number of cells was expressed as cells per ml. The count was made both in the inoculation and the control tubes.

Mitotic index The growth rate was estimated by means of the mitotic index. The mitotic index of the cultures was examined by counting the number of cells in mitosis in a representative 1000 cells and was expressed as a percentage value. Methanol fixed cultures stained with haematoxylin-eosin were used (*Paul* 1970)

Virus The virus used originated from an isolation from a cat with clinically diagnosed panleukopaenia (*Flagstad* 1973) The virus had been passed once in a kitten which died from panleukopaenia. The fourth cell culture passage of the isolation from this kitten was used for the examinations. The virus was stored at −22 C.

Cytopathic effect Examination of the cytopathic effect was made by microscopy of Leighton tubes without any staining of the cells. A culture was regarded positive for cytopathic change when both a roughening and thinning of the cell sheet could be observed. The thickness of the cell sheets was compared in control and inoculated tubes by microscopy and by cell counting

Virus inoculation Nearly all the medium was removed from the Leighton tubes and virus was inoculated. After adsorption of the virus to the cells for one hour at room temperature, 2 ml of medium was added.

Virus titration The titre of the stored virus was, under optimal conditions, $10^{5.8}$ $TCID_{50}$/ml. For determination of the titre inoculation was carried out 4 hours after seeding of the cells. The titre was determined three days after inoculation. Inoculated and control tubes were fixed in methanol and stained with haematoxylin-eosin. The stained cultures were examined for intranuclear inclusion bodies.

Susceptibility The degree of susceptibility of cultures was estimated by the maximal percentage number of cells with inclusion bodies. As the inclusion bodies were not uniformly distributed, it was necessary to examine a considerable area of the cell sheet to get reproducible results. The percentage factor was obtained by counting the total number of cells in one representative field, using 500 × magnification, and then counting the inclusion bodies in 25 such fields, five taken at each corner of the monolayer and five across the centre of the monolayer as described by *Johnson* (1965)

EXPERIMENTS AND RESULTS

Examination of the cell growth curve The cells were seeded in about 2 million per ml, 1 million per ml, 500 000 per ml, and 250,000 per ml, and the number of cells and the mitotic index were recorded daily for seven days. The cell count and recording of mitosis were made twice each for seeding of 2 million cells per ml and 500,000 cells per ml, and three to four times each when 1 million cells per ml were used. However the cell count for seeding of 1 million cells per ml two and four hours after seeding was made ten times in order to obtain experience in judging the growth intensity of cultures shortly after seeding

Using 2 million cells per ml (or more) a rapid increase in the cell growth curve to a high level viz. 4 million per ml, was seen one day after seeding after which time number of cells decreased to about 2 million per ml two days after seeding (Fig 1)

Using about 1 million cells per ml a gradual rise occurred in the cell growth curve to about 1 million per ml one day after seeding followed by a flattening of the curve (Fig 1)

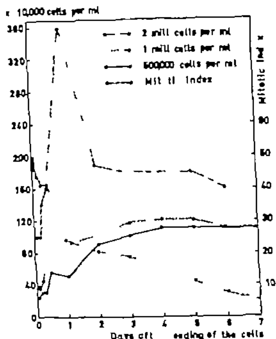


Fig 1 Cell growth curve and mitotic index for feline kidney cells seeded in a quantity of 2 million cells per ml, 1 million cells per ml, and 500,000 cells per ml.

However almost the same curve was seen when 500 000 cells per ml were used. The maximum number of cell obtained, viz. about 1 million per ml, was reached somewhat more slowly (Fig 1)

With a cell count of about 250,000 cells per ml, the cell growth was poor and irregular and therefore that type of cell culture was not used in the study

The values observed for the mitotic index were almost the same when seeding 2 million cells per ml, 1 million cells per ml, and 500,000 cells per ml. The mitotic index varied between 50 per cent and 90 per cent within 24 hours after seeding After that, a gradual fall in the mitotic index took place during the next six days to a value of about 5 per cent (Fig. 1) The curves in Fig. 1 represent average values of the results obtained in the different experiments. The curve representing the percentage of mitosis is an average of the values found in the cultures seeded with either 2 million cells per ml,

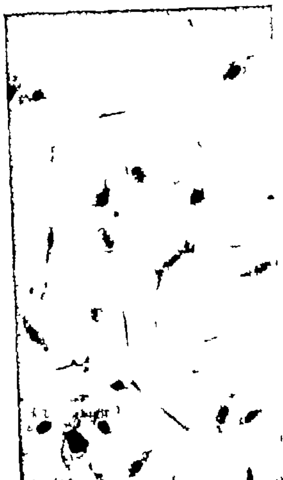


Fig 2 Feline kidney cell culture three days after inoculation. Haematoxylin eosin stain (magnification 160 ×)

1 million cells per ml, or 500 000 cells per ml, since the results obtained in the different groups seemed to be nearly identical.

Examination of the transience of the changes caused by the virus The transient nature of the cytopathic effect and the intranuclear inclusion bodies were examined by inoculation of Leighton tubes four hours, three days and six days after seeding 0.3 ml of virus was used for inoculation, the number of cells being 1 million per ml. The examinations were carried out daily for a period of ten days.

A cytopathic effect was seen in cultures inoculated four hours after seeding. This effect was most pronounced three days after



Fig 3 Feline kidney cell culture ten days after inoculation. Haematoxylin eosin stain (magnification 160 ×)

inoculation. The cells in inoculated cultures were somewhat irregular in shape and the granules of the nuclei were more distinct than in the control culture cells. The cell sheet was thinner in the inoculated cultures than in the control cultures, where it was confluent (Fig 2, Fig 4) The number of cells in the control tubes increased gradually from about 300,000 cells per ml four hours after seeding to a confluent cell sheet with about 1 million cells per ml two to three days after seeding Inoculation with panleukopaenia virus apparently resulted in lack of cell growth. No increase in the number of cells was observed during the first three to four days after seeding There were 300,000 cells per ml four hours after seeding and about 500 000 cells per ml three to four days after seeding (Fig 5) After that time an increase in the number was gradually seen in the inoculated tubes. Later it was not possible to distinguish between inoculated (Fig 3) and control cultures. Both in the inoculated and control cultures the cell sheet was rough and somewhat clumpy due to cell degeneration. The cell count was almost the same in the inoculated and the control cultures (Fig. 5)

The peak of the infection expressed as percentage of cells with inclusion bodies was seen three days after inoculation both for cell cultures inoculated four hours, three days and six days after seeding (Fig 6) The dif



Fig 4 Non-inoculated feline kidney cell culture three days after inoculation. Haematoxylin eosin stain (magnification 160 ×)

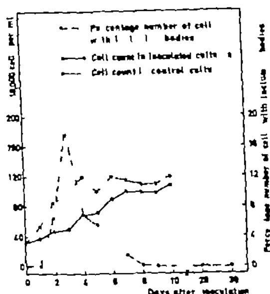


Fig 5 Examination of the transience of changes caused by the virus. Cell count and examination of percentage number of cells with inclusion bodies during ten days. 1 million cells per ml. Inoculation four hours after seeding.

The cell sheet was confluent in the first group containing 2 million cells per ml, at the time of inoculation in the period two to ten hours after seeding Inoculation during that period resulted in a decrease in the number of cells seen microscopically In the control tubes the cell sheet was confluent, whereas this was not the case in the inoculated tubes (only one-half to one-third of the glass covered with cells) Inoculation later than ten hours after seeding did not result in any clear decrease in the number of cells when examined microscopically However cell counts showed a decrease in the number of cells also when inoculation was made later than ten hours after seeding (Fig. 7) The cells sheet was completely confluent at about 1 million cells per ml and therefore, it was not possible to distinguish a difference in the number of cells in the inoculated and the control cultures about 1 million cells per ml. There was no

ference in percentage number of cells with inclusion bodies from day to day was greatest when inoculation was carried out four hours after seeding. The percentage number of cells with inclusion bodies increased during the first three days and decreased from the third to fourth day The presence of inclusion bodies was studied by examining the cultures inoculated four hours after seeding for periods of 10 days, 20 days and 30 days after inoculation. They could be found within 8 to 10 days (Fig 5) The results in Figs. 5 and 6 represent the average values from two experiments each. However examination for intranuclear inclusion bodies 20 and 30 days after inoculation was made only once.

Examination of the cytopathic effect This was made by using inoculation at different times after seeding. The cell cultures were inoculated with 0.5 ml virus two, four six and ten hours after seeding and one, two three, five six and seven days later The number of cells used for seeding were about 2 million cells per ml, 1 million cells per ml and 500,000 cells per ml. The cultures were examined three days after inoculation.

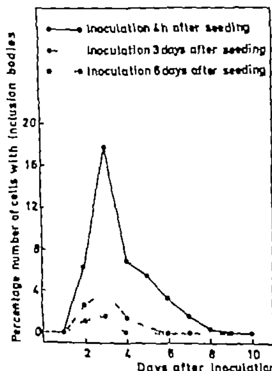


Fig 6 Examination of percentage number of cells with inclusion bodies during ten days. 1 million cells per ml Inoculation four hours, three days and six days after seeding.

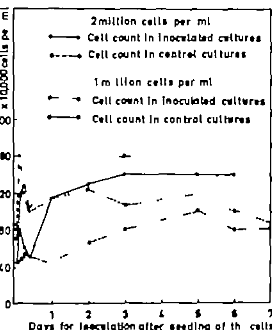


Fig 7 Cell count in inoculated and control cultures after inoculation at various times after seeding. Cell count made three days after inoculation. 1 million cells per ml and 2 million cells per ml.

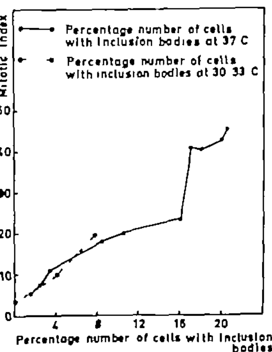


Fig 8 Percentage number of cells with inclusion bodies in relation to the mitotic index at time of inoculation at 30 to 33 and 37 C.

cytopathic change in the form of better distinction of the granules of nuclei in the inoculated cells as compared to the control cells. The latter tended to be changed in a similar way due to cell degeneration.

In the other groups, which contained 1 million cells per ml and 500,000 cells per ml at the time of seeding a cytopathic change as described above was observed until inoculation one day after seeding. When inoculation was carried out later no clear distinction could be made between inoculated and control tubes, since a roughening and clumping of the cells also occurred in the control tubes. In the cultures which are inoculated up to two to three days after seeding, it was seen clearly that the number of cells was lower in the inoculated tubes than in the control tubes. This lower number was confirmed by cell counting and could be found in the cultures which were inoculated before three to four days after seeding (Fig 7) It was found that the difference in the number of cells in the inoculated and control cultures was most pronounced when inoculation was carried out between four hours and two days after seeding (Fig 7) The results shown in Fig 7 represent average values. The cell counts from the first group, viz. 2 million cells per ml, are average values from two experiments, and the cell counts from the other groups are average values from two to nine experiments. Cell counts were made most frequently after inoculation in the period two to four hours and in the period two to three days after seeding

Examination for percentage number of cells with intranuclear inclusion bodies. This was made using inoculation at different times after seeding as described above for examination of the cytopathic effect About 2 million cells per ml, 1 million cells per ml and 500 000 cells per ml were used for seeding. The cultures were examined three days after inoculation.

The percentage number of cells with inclusion bodies was presumably independent of the number of cells seeded in the values 2 million to 500,000 cells per ml, when in-

oculated shortly after seeding in cells with high mitotic activity. When the mitotic index was about 40 per cent, the number of cells with inclusion bodies was about 15 to 20 per cent, decreasing with decreasing mitotic activity. With the mitotic index of 5 per cent, the number of cells with inclusion bodies was 1 to 2 per cent (Fig. 8). Fig. 8 represents average values from two to ten experiments. Most of the inoculation experiments were made in the period two hours to two days.

Slowly growing cells. In order to prepare slowly growing cells, some culture were incubated at 30° to 33° C. For comparison, cell cultures prepared from the same batch of cells were incubated at 37° C. This experiment was made in order to examine the influence of the mitotic activity on virus multiplication. Inoculation with 0.3 ml virus was carried out four hours, three days and seven days after seeding, using 1 million cells per ml. The cultures were examined three days after inoculation.

Growth of the cultures at 37° C resulted in rapidly growing cells with high mitotic activity and a high percentage of cells with inclusion bodies, as also described earlier for cells grown at 37° C (Fig. 1, Fig. 8). Incubation at 30° to 33° C resulted in slow growth of the cell cultures, with lower mitotic index than in the cells grown at 37° C. No cytopathic effect and no decrease in the number of cells were observed at the lower temperature. After inoculation in one-day old cultures, slightly lower number of cells was observed microscopically and by cell count, in comparison with the number of cells in the control cultures. The cells in the control tubes were scattered and only half of the glass was covered with cells. It was therefore difficult to observe a lower number of cells in the inoculated tubes by microscopy. In addition, the cell count was so low that the relative error in the counting procedure was great. The percentage number of cells with inclusion bodies was lower when the mitotic index was lower (Fig. 8).

Influence of different serum supplements on virus multiplication. In addition to the examinations described above, the influence of different serum supplements on the virus was studied. The medium for cell cultures was supplemented either with 10 per cent foetal bovine serum (Flow Laboratories) or with 10 per cent calf serum (Flow Laboratories L40318). Titration with 0.1 ml virus was carried out four hours after seeding, using 1 million cells per ml. The cultures were examined three days after inoculation.

Using foetal bovine serum as supplement for the cell cultures, the titre of the virus was found to be 10[illegible] $TCID_{50}$/ml, while when using calf serum the titre was 10[illegible] $TCID_{50}$/ml. A cytopathic effect as described above was seen when foetal bovine serum was used, but not when calf serum was used. The number of cells with inclusion bodies was 12.8 and 3.8 per cent, respectively.

DISCUSSION

In the present study it was seen that when the mitotic index was high, the manifestation of the virus infection, expressed as percentage number of cells with inclusion bodies, was correspondingly high. When the mitotic index was low, the percentage number of cells with inclusion bodies was also low. It was further found that there was agreement in the results obtained using cultures grown at 37° C and those grown at 30° to 33° C. With the same mitotic index, almost the same percentage number of cells with inclusion bodies was counted.

Johnson (1967) has demonstrated „that the virus has a specific affinity for cells in active mitosis". Primary cell cultures were used in Johnson's study. In order to obtain high susceptibility, inoculation was carried out when the cell sheet was a quarter to half complete. Secondary cell cultures were used in the present study, since it was found that they grew more uniformly than primary cells. The percentage number of cells with inclusion bodies in relation to various phases of the cell cultures found in this study agrees with the findings of *Johnson*. Using secondary cultures, the in vitro examination of

the feline panleukopaenia virus could be carried out with regularity and the results obtained were reproducible. Further passage of the cell cultures—particularly after three to four passages—resulted in slow growth of cells apparently not suitable for supporting virus multiplication.

It was found that the best cultures for virus multiplication were those seeded in a quantity of about 1 million cells per ml to 500 000 cells per ml.

A cytopathic effect was seen in cell cultures seeded with 1 million to 500,000 cells per ml if inoculation was carried out during the first 24 hours after seeding using inoculation og 0 1 ml of a virus sample with titre $10^{3.5}$ $TCID_{50}$/ml.

The metabolism was great in cell cultures seeded with 2 million cells per ml and degeneration of the cells occurred early after seeding. This may be the reason why no cytopathic effect was seen in these cultures, except for a lower number of cells in the inoculated tubes than in the control tubes.

A lower number of cells was found by inoculation for a longer period in cell cultures seeded in a quantity of 1 million cells per ml than in cell cultures seeded in a quantity of 2 million cells per ml. This difference in the number of cells in inoculated tubes in comparison with the control tubes is caused probably by lack of cell growth after inoculation with the panleukopaenia virus.

The panleukopaenia virus isolated by *Gorham et al.* (1966) had a cytopathic effect where degeneration was in process and infected cells sloughed from the glass. They suggested that the reason for this was "other presently unknown virus which were carried with their panleukopaenia virus passages". The changes caused by the panleukopaenia virus isolated by *Johnson* (1965) *King & Croghan* (1965) and *Scott et al.* (1970) was of a transient *nature like the virus described* above.

In the present study a heavy dose of virus was used with the intention of examining the occurrence of the cytopathic effect. Using such a dose, the cytopathic effect was most pronounced three days after inoculation. Therefore examination of the cytopathic effect and the percentage number of cells with *inclusion bodies* was made three days after inoculation. Later the cytopathic effect disappeared, leaving a full sheet of cells, even in cases in which a marked cytopathic effect had been observed previously (Fig. 2, Fig 3). The percentage number of cells with inclusion bodies was also greatest three days after inoculation, after which time they gradually disappeared, and about ten days after inoculation no inclusion bodies were found.

An inhibitory effect of calf serum (Flow Laboratories L40318) on the growth of the virus has been observed in this study. A similar effect was observed previously using different calf sera. This inhibitory effect of some calf sera has also been reported by *Johnson* (1967).

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REFERENCES

Flagstad A., A study on in vitro isolation of feline panleukopaenia virus. Acta path. microbiol. scand. Sect. B, *81* 385 392 1973.

Gorham J R., Hartsough G R., Sato N. & Lust S Studies on cell culture adapted feline panleukopenia virus—neutralization and antigenic extinction. Vet. med *61* 35–40, 1966.

Johnson R H Feline panleucopaenia virus II Res. Vet. Sci. 6 472–481 1965

Johnson R H., Feline panleucopaenia virus IV Res. Vet. Sci. *8* 256–264 1967

King, D A & Croghan D L., Immunofluorescence of feline panleucopaenia virus in cell culture. Determination of immunological status of felines by serum neutralization. Canad. J comp. Med. Vet. Sci. *29* 85–89 1965.

Madin S H., Andersen P C & Darby N B. The in vitro cultivation of tissues of domestic and laboratory animals. Amer J Vet. Res. *18* 932 941 1957

Paul J Cell and tissue culture 4th Edition, E. & S. Livingstone Great Britain, 1970, pp. 323 324

Scott F., Csiza C. K. & Gillespie J H Feline viruses. V Cornell Vet. *60* 183–192, 1970

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LOCALIZATION OF ANTIGENS IN THIN SECTIONS OF BACTERIA BY THE IMMUNO-PEROXIDASE TECHNIQUE

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Haugen, A., Helgeland, S. & Grov A. Localization of antigens in thin sections of bacteria by the immuno-peroxidase technique. Acta path. microbiol. scand. Sect. B, 83 79–90 1975.

The immunoperoxidase technique together with electron microscopy has been examined for the ultrastructural localization of staphylococcal protein A, the immunoferritin method being included for comparison. The results show that protein A is uniformly distributed in the whole cell wall. Both the direct and indirect methods, Fab- as well as Fc-reactions, showed identical results. The immunoperoxidase method was superior to the immunoferritin method, especially when applied to thin sections of the bacteria, and the clear specificity demonstrated indicates a useful method for localization of cellular antigens.

Key words Antigen localization immuno-peroxidase technique bacteria.

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The immuno-enzyme technique was introduced in 1966 (4 6 33 34) for localization of cellular components. Of the enzymes employed horseradish peroxidase (1 25 31) alkaline phosphatase (22 33 34 41) glucose oxidase (1) and tyrosinase (2) the first one seems to be most profitable. Horseradish peroxidase is very resistant to various histological operations and is easily detected by 3,3'-diaminobenzidine which oxidizes in the presence of H_2O_2 to a phenazine polymer This dark-brown reaction product is reactive to OsO_4, the resultant product being visible in a light or electron-microscope (12)

The technique has found widespread application (3 17 29 32, 46) owing to its specificity and high sensitivity Applied to localization of antigens on thin sections it has, however been of limited value (17 22, 29) and to our knowledge no report has appeared dealing with immuno-enzyme tracing of antigens on thin sections of bacteria.

The intention of the present study was to examine the immunoperoxidase technique for electronmicroscopic studies of antigens on thin sections of bacteria in comparison with the immunoferritin method (43) For this purpose the staphylococcal antigen protein A (13) was chosen since both Fab-reactivity of specific antibodies and Fc-reactivity of normal human IgG could be utilized (13)

MATERIALS AND METHODS

Embedding Media

Butyl methyl- and hydroxypropyl methacrylate and Epon 812 were purchased from Koch-Light, England; glycolmethacrylate from Serva, BRD

Serum albumin fraction V from Armour Laboratories, England and the Spurr (epoxy) medium was from Taab Laboratories, England.

Enzymes

Papain (2 × cryst., cat. no. P3125) pepsin (2 × cryst., cat. no. P6875) and horseradish peroxidase (RZ1 5 P8250) were all obtained from Sigma, USA.

Chemicals

Agar Noble, Bacto peptone, Bacto beef extract, and Freund's adjuvant were purchased from Difco Laboratories, USA diethylamino ethyl (DEAE) cellulose from Eastman Organic Chemicals Department, USA, Sephadex and Sepharose from Pharmacia, Sweden and ferritin (2 × cryst.) propyleneoxide and glutaraldehyde from Serva. Paraformaldehyde was from BDH, England acrolein from Baker Organic Chemical, Holland α, α' azoisobutylnitril from Koch-Light and γG-Kabi (Cohn fraction II) from Kabi, Sweden.

Bacterial St ains

Two strains of *Staphylococcus aureus* Cowan I (NCTC 8530) (36) and Wood 46 (NCTC 10344) (15) were employed, the latter strain being protein A negative.

Growth and Harvesting

The bacteria were grown on broth (0.3 g beef extract, 1 g peptone and 0.5 g of NaCl per litre) harvested at the end of the exponential growth-phase and washed twice in phosphate-buffered saline (PBS) by centrifugation at 2000 × g in the cold for 15 min.

Protein A was isolated as described in (14)

IgG was precipitated from sera in 33 per cent $(NH_4)_2SO_4$ and purified on columns of DEAE-Sephadex and/or DEAE-cellulose (9) The source of human IgG was a pool of normal sera from healthy blood donors. Specific antibodies to human IgG were isolated on an immunoadsorbent column of γG-Kabi coupled to Sepharose 4B activated by cyanogen bromide (37) Bound material was eluted with 3 M NaSCN (40)

$F(ab')_2$-fragments of IgG were isolated from pepsin-digests (10) on columns of Sephadex G-150 (2.8 × 85 cm) and *Fc-fragments* of IgG from papain-digests on columns of DEAE-cellulose (38) Solutions of IgG/fragments were concentrated by vacuum dialysis (Sartorius membrane filter GmbH, Göttingen, BRD) the purity being controlled by immunoelectrophoresis using specific antisera, and quantitated by a modified Folin-Ciocalteau method (24)

Antisera

Antibodies to protein A were raised in rabbits by injection of 100 μg in 0.25 ml of saline emulsified in an equal volume of Freund's complete adjuvant (FCA) into the hind foot-pads, and an equal dose intramuscularly (i.m.) after 4 weeks. The animals were bled 3 weeks later. Anti-human IgG antibodies were produced in rabbits receiving 3 mg IgG in FCA in one foot-pad, an equal dose subcutaneously 5 weeks later and finally an intravenous injection of 2.5 mg IgG in 1 ml of PBS. The animals were bled one week later Antiserum to rabbit IgG was produced in a goat given 4 i.m. injections, one week apart, of 3 mg IgG in 0.5 ml saline mixed with 0.5 ml of FCA, and a fifth injection of the same dose 2 months later Antiserum to $F(ab')_2$ from rabbit IgG was produced in guinea pigs by injecting 2.5 mg $F(ab')_2$ in 0.5 ml PBS mixed with 0.5 ml FCA into both hind foot-pads. Further i.m. injections were given 4 and 7 weeks later and the animals were bled out 5–10 days after the last injection.

Goat antisera to human Fab- and Fc-fragments were obtained from Hyland, Belgium.

Peroxidase-labelling of IgG and IgG-fragments

Peroxidase-conjugated IgG and Fc-fragments were prepared according to (2) whereas $F(ab')_2$ conjugates were prepared according to (5) The serological and enzymatic activity of the conjugates was controlled in connection with immunoelectrophoresis, showing that the precipitin lines formed with specific antisera to IgG/fragments were coloured after incubation with H_2O_2-diaminobenzidine.

Ferritin-labelling of IgG was done according to (19)

Immunoelectrophoresis was carried out in 1 per cent agar Noble in sodium veronal buffer pH 8.7 I 0.05 for 1 h at 10 V/cm

Electron microscopic studies were carried out on a Philips 300 electron microscope at 80 KV

Preparation of Samples for Electron Microscope Study

For morphological studies the procedure of *Kellenberger et al.* (18) was followed using Epon 812 as embedding medium.

Samples treated with human IgG were prepared as follows:

Two ml of human IgG solutions ranging from 10 to 100 μg/ml were added to 2 ml of bacterial suspension (10^9 bacteria/ml) incubated at room temperature for 45 min and washed 3 × in PBS The bacterial sediment was fixed for 30 min with 2.5 per cent glutaraldehyde in 0.2 M Cacodylate buffer pH 7.3 (39) washed 3 × in the same buffer postfixed for 2 h with per cent OsO

the same buffer and dehydrated and embedded Epon 812 (18). For studies after treatment of bacterial surface with peroxidase-conjugated and ferritin-conjugated IgG (indirect method) the following procedure was used. To samples of bacterial cellsediment from 5 ml suspension (10^9 bacteria/ml) washed in PBS were added 1 ml portions of human IgG ranging from 10 to 100 µg/ml. After 45 min at room temperature and three washings in PBS the cells were resuspended in 0.3 ml of conjugated IgG from rabbit anti-human IgG and further incubated for 45 min at room temperature. (This incubation time was found to be optimal after several time intervals had been tried). The cells were then thoroughly washed and fixed for 30 min with 2.5 per cent glutaraldehyde in 0.1 M phosphate buffer pH 7.2, and washed overnight in the same buffer. Bacteria treated with ferritin conjugate were fixed as above and then treated according to (18). The bacteria treated with peroxidase-conjugated IgG were added to H_2O_2-diaminobenzidine solution (12), thoroughly washed in PBS, suspended in 1 per cent agar, cut into cubes (1 mm) and further fixed with OsO_4, dehydrated and embedded in Epon 812 (18).

Fixation

The following fixatives (diluted in 0.1 M phosphate buffer pH 7.3) and fixation times were tested:

a) Paraformaldehyde (3 per cent) for 15–30 min.

b) Paraformaldehyde (3 per cent) + acrolein (1 per cent) for 15–30 min.

c) Paraformaldehyde (1 per cent) + acrolein (1 per cent) for 30 min.

d) Paraformaldehyde (1 per cent) + glutaraldehyde (1 per cent) for 15–30 min.

e) Glutaraldehyde (1 per cent) for 15–30 min.

Embedding

The following embedding media were tested: Methacrylate (butylmethacrylate-methylmethacrylate, 4:1) (11), glycolmethacrylate (20), hydroxypropylmethacrylate (21), Epon 812 (18), Spurr (45) and bovine serum albumin (BSA) (27).

Localization of Antigen (Protein A) on Thin Sections of Bacteria Embedded in Spurr

Embedded bacteria were cut into thin sections of approximately 80 nm (estimated by reflection of light) on an Ultramicrotome (LKB produkter, Sweden) and the thin sections were transferred to grids (300 mesh, formvar coated). The Spurr medium was etched by floating the grids on drops of fresh aqueous 10 per cent H_2O_2 for 10 min. The grids were then washed in 3 beakers of distilled deionized water and subsequently incubated with sera and/or conjugate. The sera and conjugates were diluted to appropriate concentrations in Tris-buffered saline (TBS): 0.5 M Tris-HCl-buffer pH 7.6, diluted 1:10 with 0.9 per cent NaCl, just before use.

The grids were either floated for 10–15 min on drops of human IgG (concentrations from 10 to 100 µg/ml were tested), washed 3 × in TBS and then floated on drops of conjugate for 10 min followed by 3 washings in TBS (indirect method) or transferred directly to drops of conjugate (direct method). Immediately thereafter, staining was performed with the following solution: 20 mg diaminobenzidine added to 175 ml of 0.05 M Tris-HCl, pH 7.6, containing 1.5 ml of 0.3 per cent H_2O_2, and filtered on glass-filter (G 4, Schott & Gen., Mainz, BRD). The grids were mounted on forceps and dipped for 7–10 min into the solution, which was held in movement by a magnetic stirrer. After thorough washing in distilled water the grids were airdried, floated in drops of 1 per cent OsO_4 for 20 min, and washed in distilled water. Sections with methacrylate and Epon as embedding materials were treated according to *Kawarai & Nakane* (17). As for the Spurr medium, the Epon sections were etched by H_2O_2 prior to immune reactions, whereas in the methacrylate sections a part of the embedding material was removed by water-saturated solutions of xylene. Sections with glycolmethacrylate and hydroxypropylmethacrylate were incubated with antibodies and/or conjugates without prior etching. The incubation time was varied between 30 and 60 min as described in (20, 21). The sections with BSA were treated with a 4 per cent solution of BSA in PBS, washed, incubated with antibodies and/or conjugates for 5 min, and then continued according to (27).

Control of Specificity

To test for specific reaction with protein A, sera (IgG-solution) and conjugates were absorbed with protein A prior to use on thin sections. In addition, the reagents were tested on thin sections of the protein A negative strain Wood 46. Furthermore, exclusion of antiserum to protein A (Fab-reaction) or IgG of human serum (Fc-reaction) was used to test the conjugates in the indirect method.

Tracing with Ferritin-conjugated IgG on Thin Sections

Thin sections of bacteria in glycolmethacrylate and hydroxypropylmethacrylate were treated with sera and conjugate at various time-intervals of incubation. Prior to incubation with the ferritin-conjugate the grids were lowered into a small vessel

containing about 1 ml of PBS. Two drops of conjugate were added, and after appropriate incubation time the unreacted conjugate was washed off by PBS. Thin sections of bacteria in BSA were treated as described by *McLean & Singer* (28).

Use of Tissue powder to Remove Unspecific Tracing of Thin Sections

To avoid unspecific reactions with serum components, homogenized rat liver powder (100 mg) moistened with TBS was added to serum (1 ml diluted to 10 ml with TBS), incubated with stirring for 2 h and centrifuged to remove the powder (30).

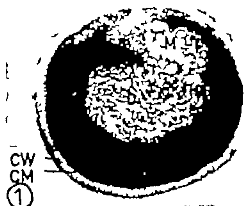


Fig. 1. Electron-micrograph of *S. aureus* strain Cowan I embedded in Epon 812 showing cell wall (CW), cell membrane (CM), DNA-threads (D) and mesosome structures (M). × 60,000.

RESULTS

The cell morphology was found to be identical for the two strains of *S. aureus*, Wood 46 and Cowan I (Fig. 1). However, after incubation with human IgG and staining with uranylacetate, the two strains showed certain differences (Fig. 2). The cell wall of Cowan I, but not of Wood 46 bacteria, had become coated with a fluffy layer of IgG molecules. Incubation with peroxidase labelled anti-human IgG after prior treatment of whole bacteria with human IgG showed a homogeneous tracing of the Cowan I cell wall towards the cell membrane (Fig. 3). This indicates that both human IgG and peroxidase labelled rabbit IgG penetrate the bacterial cell wall and also that protein A is uniformly distributed in the whole cell wall. In contrast, only a superficial tracing of Cowan I bacteria was observed (Fig. 4) when ferritin-conjugated anti-human IgG was used.

Protein A was found to retain its activity for human IgG after exposure to the various fixatives. On the contrary, the embedding media and polymerization procedure appeared to influence the reactivity. Of the embedding media and fixatives tested, fixation in glutaraldehyde and/or paraformaldehyde combined with embedding in Spurr medium was found to give the best results with regard to morphology as well as to the reactivity of protein A. Optimal conditions for etching were found to be in 10 per cent H_2O_2 for 10 min. Etching with H_2O_2 of thin sections containing Epon 812 usually leads

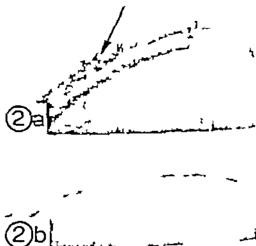
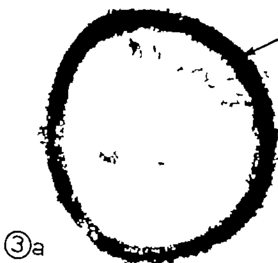


Fig. 2. S. aureus Cowan I (a) and Wood 46 (b) cells incubated with human IgG (100 µg/ml) and embedded in Epon 812. The arrow indicates the fluffy layer of IgG distributed over the entire surface of Cowan I. × 150 000 (a), × 140,000 (b).

to deformation of the cell morphology or destruction of specimen. In those specimens that were intact the tracing was uneven (Fig. 5). Optimal time of reaction with specific antiserum and/or conjugate was found to be about 10 min. Longer incubation did not increase the specific tracing but gave an unspecific background tracing. However, at





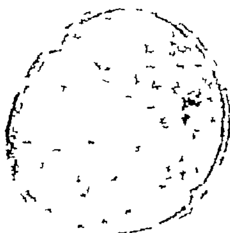


Fig 3 S aureus Cowan I (a) and Wood 46 (b) lls incubated with human IgG followed by peroxidase-labelled rabbit anti-human IgG embedded in Epon 812 Note the uniform distribution of peroxidase reaction product in the Cowan I cell wall arrow) × 65,000



Fig 4 S aureus Cowan I (a) and Wood 46 (b) incubated with human IgG and rabbit anti-human IgG labelled with ferritin and embedded in Epon 812 × 65,000

Fig 5 S aureus Cowan I ells fixed in 3 per cent utaformaldehyde and embedded in Epon 812. The thin sections ere etched with 10 per cent H_2O or 10 min, incubated with human IgG followed by eroxidase-conjugated rabbit anti-human IgG H_2O -diaminobenzidine and OsO treatment. × 0,000



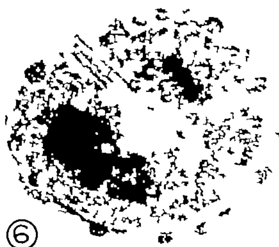


Fig. 6. Thin sections of *S. aureus* Cowan I fixed in 3 per cent paraformaldehyde, embedded in BSA and incubated with human IgG. Unspecific deposits of peroxidase reaction product are seen after treatment with peroxidase-labelled rabbit anti-human IgG, H_2O_2-diaminobenzidine and OsO_4. × 65,000



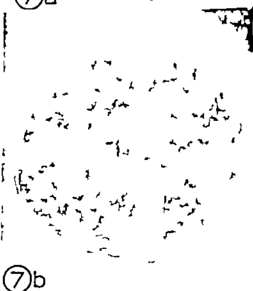


Fig. 7. S. aureus Cowan I (a) and Wood 46 (b) cells fixed in 3 per cent paraformaldehyde and embedded in Spurr medium. Thin sections were etched with H_2O_2, incubated with human IgG followed by peroxidase-labelled rabbit anti-human IgG. A uniform distribution of peroxidase reaction product in the Cowan I cell wall is noted after treatment with H_2O_2-diaminobenzidine and OsO_4. × 70 000



Fig. 8. S. aureus Cowan I cells embedded in BSA. Thin sections were incubated with human IgG followed by ferritin-labelled rabbit anti-human IgG. Note the unspecific tracing obtained. × 55,000

sorption of sera and conjugates with the rat liver homogenate reduced the background to a minimum. A lower concentration of the H_2O_2-diaminobenzidine reagent than d... scribed in (12) and movement of the sol... tion during incubation of the grids, also r... duced the background.

The results using methacrylate hydrox... propyl-methacrylate glycolmethacrylate ar...

9a

10a

9b

10b

10c

9 This sections of S aur u Cowan I cells ned in 3 per cent paraformaldehyde and embedled in Spurr medium. a. shows the result when ncubation with human IgG is omitted b when the gG-solution used for incubation is absorbed with rotein A × 75,000 (a) × 65,000 (b)

10 S au eu Cowan I (a) and Wood 46 (b) ll fixed in 3 per ent paraformaldehyde and embedded in Spurr medium Thin sections were treat d with H O followed by human Fc-peroxidase onjugate and then th H O -diaminobenzidine nd OsO Note the uniform distribution of peroxiase reaction product in the Cowan I cell wall show section of Cowan I cell treated simil but with F -conjugate which was bsorbed h protein A 63,000

bovine serum albumin as embedding media were unsatisfactory. Unspecific deposits dominated the electron micrographs (Fig. 6). Variation in the time of incubation of methacrylate-sections in water-saturated solutions of xylene between 2 and 20 min, different amounts of water in glycolmethacrylate and hydroxypropylmethacrylate, variation in time and incubation of sera and/or conjugates (3 min–16 h) and various polymerization procedures (UV light and heat) were all without success. Bacteria embedded in the methacrylate materials were deformed during the polymerization procedure.

Thin sections of Cowan I and Wood 46 embedded in the Spurr medium appeared to have intact morphology and the indirect peroxidase tracing showed IgG-reactive structures uniformly distributed in the cell wall of strain Cowan I in agreement with the results obtained with whole cells. No tracing could be observed on the thin sections of Wood 46 cells (Fig. 7). Use of ferritin-conjugate on thin sections gave too much unspecific tracing (Fig. 8). The specificity of the reactions with peroxidase conjugates was shown by exclusion of human IgG in the indirect method as well as by absorption of the IgG-solution with protein A prior to incubation (Fig. 9) after which no tracing was observed.

The direct method with peroxidase-conjugated human Fc and $F(ab')_2$ from rabbit anti-protein A IgG showed the same result as in the indirect method, a homogeneous tracing of the cell wall of Cowan I cells (Figs. 10a and 11a) but no tracing of the sections of Wood 46 cells (Figs. 10b and 11b). The reactivity of the conjugates du-



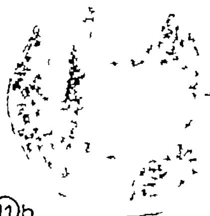




Fig. 11. S. aureus Cowan I (a) and Wood 46 (b) cell fixed in 3 per cent paraformaldehyde and embedded in Spurr medium. Thin sections were treated with H_2O_2 followed by rabbit anti-protein A $F(ab')_2$-peroxidase conjugate and then H_2O_2 diaminobenzidine and OsO_4. Note the uniform distribution of peroxidase reaction product in the Cowan I cell. 11 C shows a section of a Cowan I cell treated similarly but with a $F(ab')_2$-conjugate which was absorbed with protein A. × 70 000.

appeared upon absorption with protein A (Figs. 10c and 11c).

DISCUSSION

Incubation of *S. aureus* cells with human IgG solutions clearly differentiated the protein A positive Cowan I strain from the protein A negative Wood 46 strain. In agreement with the finding of *Nickerson et al.* (35) the Cowan I strain became coated with a layer of IgG. This indicates that protein A is ultrastructurally located on the outermost portion of the cell wall only. However the immunoperoxidase technique applied to whole cells showed a uniform staining of the whole cell wall of protein A positive cells. Similarly *Wagner & Wagner* (46) showed the M protein of *Streptococcus pyogenes* to be distributed in the cell wall. The use of ferritin-conjugated IgG resulted in a superficial staining only. Thus, the use of ferritin-conjugates led *Lind et al.* (25) to conclude that protein A was situated at the outer part of the cell wall. The demonstration by peroxidase-conjugates of an even distribution of protein A structures all over the cell wall was further confirmed using Fc and $F(ab')_2$-conjugates on thin sections of the bacteria. The difference between the peroxidase and the ferritin techniques on whole cells is most probably due to the difference in molecular weight of peroxidase and ferritin, approx. 40,000 and 750,000 respectively. Peroxidase-conjugated IgG molecules are apparently still able to penetrate the cell wall.

Whereas peroxidase-conjugate gives a continuous and homogeneous staining of the cell wall, the staining with ferritin-conjugate is erratic. *Bretton et al.* (7) have shown by double tracing with ferritin and peroxidase-conjugate on the same preparation that peroxidase-conjugate apparently traces more antigenic sites than ferritin-conjugate. This is most probably due to steric hindrances of the relatively large ferritin molecules.

In most of the previous studies with the immuno-peroxidase and immuno-ferritin method on cellular antigens, tracing has been performed prior to embedding of the cells. To our knowledge this is the first paper in which the peroxidase technique is used to localize an antigen directly on thin sections of bacteria. The advantage of this method is that the conjugate may react directly with an intracellular antigen without prior disintegration of cell wall and cell membrane, and also that several tests may be carried out on the same embedded cells.

Fixation with glutaraldehyde has been found to inactivate the antigenic sites of proteins (42). In the present study the activity was retained after fixation with glutaraldehyde but thorough washing to remove traces of the fixative seemed necessary. The time of fixation should be as short as possible but long enough to avoid structural changes and loss of components of the specimen. In our experiments 30 min fixation was found to be optimal. No difference in the immunological activity was observed using glutaraldehyde, paraformaldehyde or both. The best maintenance of the morphology of the bacteria was found by the use of glutaraldehyde. Fixation with acrolein (8, 9) was not found to give better tracing.

Brief fixation of the bacteria resulted in a light cell wall of low electron-density even after positive staining with uranyl-acetate and/or lead citrate. This is in accord with previous observations (16, 42) and the reason may be that after fixation with aldehyde only materials containing RNA are stained (26). This may be the explanation of the strong intracellular staining with lead citrate, and, would lead to the conclusion that only uranylacetate should be used for detection of intracellular antigens.

The Spurr medium was found most suitable as embedding material for studies of thin sections of bacteria. Dehydration, embedding and etching of the embedding material with H_2O_2 did not show any influence on the reactivity of protein A, either with the Fc part of human IgG or with the specific $F(ab')_2$ of rabbit anti-protein A IgG. It must, however be stressed that protein A is a relatively stable polypeptide and it can

automatically be concluded that this procedure is applicable to all types of antigens.

Questions have been raised (44) about the use of epoxy monomers, e.g. Epon and Spurr as embedding materials for localization of antigens on thin sections. It is supposed that proteins and other macromolecules are easily modified by reactions with such substances. On the other hand, epoxy polymers have turned out to be most suitable for maintaining ultrastructural morphology.

The present results show that it is possible to expose intact antigens in the Spurr medium by etching with H_2O. Similar etching has been used to detect tissue-antigens after embedding in Epon 812 but this material proved unsuitable for localization of bacterial antigens. The water-soluble embedding polymers were, owing to the exclusion of the dehydration procedure, expected to effect little or no conformational changes of the protein structure. However the high degree of unspecific tracing and a frequent fragmentation of the cells during thin-sectioning make these polymers inapplicable. Generally specimens embedded in methacrylate materials often showed polymerization damage which also has been the experience of others (Dr *M. S. C. Birbeck*, personal communication). The peroxidase conjugates showed no unspecific affinity to the Spurr medium, neither was the reaction product after addition of H_2O_2-diaminobenzidine fixed unspecifically when the reaction was carried out in a continuous stream of the solution.

Ferritin-conjugated immunoglobulins have been found useless in localization of antigens on thin sections owing to unspecific adsorption to the polymer (44) and this was also the case in the present experiments with each of the embedding materials included.

In *S. aureus* protein A is the only antigen reacting with the Fc portion of human IgG. Thus, peroxidase-conjugated human Fc shows only protein A. The results obtained clearly show that protein A is uniformly distributed in the whole cell wall.

Providing that the antigens do not inactivate during embedding of the cells and preparation of thin sections and also that antibodies against the antigen can be produced and isolated the technique described should be highly suitable for localization of antigens on thin sections, and thereby facilitate studies of various problems within cell biology.

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REFERENCES

1. *Avrameas, S.*: Détection d'anticorps et d'antigènes à l'aide d'enzymes. Bull. Soc. Chim. biol. *50*, 1169–1178, 1968.
2. *Avrameas, S.*: Coupling of enzymes to proteins with glutaraldehyde. Use of the conjugates for the detection of antigens and antibodies. Immunochemistry 6, 43–52, 1969.
3. *Avrameas, S.*: Immunoenzyme techniques: enzymes as markers for the localization of antigens and antibodies. Int. Rev. Cytol. *27*, 349–385, 1970.
4. *Avrameas, S. & Lespinats, G.*: Enzymes couplées aux protéines: leur utilisation pour la détection des antigènes et des anticorps. C. R. Acad. Sci. *265*, 1149–1152, 1967.
5. *Avrameas, S. & Ternynck, T.*: Peroxidase labelled antibody and Fab conjugates with enhanced intracellular penetration. Immunochemistry *8*, 1175–1179, 1971.
6. *Avrameas, S. & Uriel, J.*: Méthode de marquage d'antigènes et d'anticorps avec des enzymes et son application en immunodiffusion. C. R. Acad. Sci. *262*, 2543–2545, 1966.
7. *Bretton, R., Ternynck, T. & Avrameas, S.*: Comparison of peroxidase and ferritin labelling of cell surface antigens. Exp. Cell Res. *71*, 145–155, 1972.
8. *Burgos, M. H., Vitale-Calpe, R. & Tellez de Iñon, M. T.*: Studies on paraformaldehyde fixation for electron microscopy. I. Effect of concentration on ultrastructure. J. Microscopie 6, 457–468, 1967.
9. *Campbell, D. H., Garvey, J. S., Cremer, N. E. & Sussdorf, D. H.*: DEAE-cellulose chromatography. In Methods in Immunology. Benjamin Inc., New York 1970, pp. 193–197.
10. *Campbell, D. H., Garvey, J. S., Cremer, N. E. & Sussdorf, D. H.*: Enzymic digestion of IgG. In Methods in Immunology. Benjamin, Inc., New York 1970, pp. 2?4–234.
11. *Dawes, C. J.*: Plastic mixture and embedding. In Biological Techniques in Electron Microscopy. Barnes & Noble, Inc., New York 1971, pp. 66–67.

Graham, R. C & Karnovsky M J The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney ultrastructural cytochemistry by a new technique. J Histochem. Cytochem. *14* 291–302, 1966.
Grov A Studies on the interaction between staphylococcal protein A and the Fc-region of Immunoglobulin G Acta path. microbiol. scand. Sect. A. Suppl. *236* 77–83 1973
Grov A Myklestad, B & Oeding P Immunochemical studies on antigen preparations from *St phylococcus au eus* I Isolation and chemical characterization of antigen A. Acta path. microbiol. scand. *61* 588–596, 1964
. *H ukenes G:* Immunochemical studies on polysaccharide A of *Staphylococcus aureus.* 2 Further studies on purification methods. Acta path. microbiol. scand. *55* 117–126 1962.
. *Huis in 'T Veld J H J & Linssen W H* The localization of streptococcal group and type antigens an electron-microscopic study using ferritin-labelled antisera J gen. Microbiol. *74* 315–324 1973
Kawarai Y & Nakane P K. Localization of tissue antigens on the ultrathin sections with peroxidase-labelled antibody method. J Histochem. Cytochem. *18* 161–166 1970
8. *Kellenberger E., Ryter A & Bichard J* Electron microscopic study of DNA-containing plasma II Vegetativ and mature phage DNA as compared with normal bacterial nucleoids in different physiological states. J biophys. biochem. Cytol. *4* 671–676 1958.
9 *Kind J & Krieg J.* Gl taraldehyde als Kopplungsreagens von Ferritin and Antikörper Cytobiologie *6* 22–28 1972
10 *Leduc E. H & Bernhard W.* Recent modifications of the glycol methacrylate embedding procedure. J Ultrastruct. Res. *19:* 196–199 1967
11 *Led c E. H & Holt S J* Hydroxypropyl methacrylate a new water-miscible embedding medium for electron microscopy J Cell. Biol *26* 137–155, 1965
22 *Led E. H Scott C B. & At aneas S* Ultrastructural localization of intracellular immune globulins in plasma cells and lymphoblasts by enzyme-labelled antibodies. J Histochem. Cytochem. *17* 211–224, 1969
23 *Lind I Reyn, A. & Bir h-Andersen A* Electron microscopy of staphylococcal protein A reactivity and specific antigen-antibody reactions. Acta path. microbiol. scand. Sect. B, *80* 281–291 1972.
24 *Lowry O H R sebrough N J., Farr A L. & Randall R L.* Protein measurement with Folin phenol reagent. J biol. Chem. *193* 265–275 1951
25 *Mandy W J & Nisonoff A.* Effect of reduction of several disulfide bonds on the properties and recombination of univalent fragments of rabbit antibody J biol. Chem. *238* 206–213 1963
26. *Marinozzi V.:* The role of fixation in electron staining. J roy micr Soc. *81:* 141–154 1963
27 *McLean J D & Singer S J* Cross-linked polyampholytes. New water-solubl embedding media for electron microscopy J Cell. Biol. *20* 518–521 1964
28 *McLean J D & Singer S J.* A general method for the specific staining of intracellular antigens with ferritin-antibody conjugates. Proc. nat. Acad. Sci. (Wash.) *65* 122–128, 1970.
29 *Moriarty G C & Halmi, N S* Adrenocorticotropin production by the intermediate lobe of the rat pituitary An electron microscopic immunohistochemical study Z. Zellforsch. *132* 1–14 1972.
30. *Naik D V.* Immunohistochemical localization of adrenocorticotropin and melanocyte stimulating hormone in pars intermedia of rat hypophysis. Z. Zellforsch. *142* 289–304 1973
31 *Nakane P K.* Simultaneous localization of multiple tissue antigens using the peroxidase-labelled antibody method A study on pituitary glands of the rat. J Histochem. Cytochem. *16* 557–560 1968.
32. *Nakane P K. Nicholds G E. & O ander D L.* Cellular localization of leucine-binding protein from *Escheri hia coli.* Science *161* 182–183 1968.
33 *Nakane P K. & Pierce G B.* Enzyme-labelled antibodies: preparation and application for the localization of antigens. J Histochem. Cytochem. *14* 929–931 1966.
34 *N kane P K. & Pierce G B.* Enzyme-labelled antibodies for the light and electron microscopic localization of tissue antigens J Cell. Biol. *33* 307–318, 1967
35 *Nickerson D S Whit J G., Kronvall, G., Williams R. C J & Quie P G* Indirect visualization of *Staphyloc ccus aureus* protein A. J exp Med. *131* 1039–1047 1970.
36. *Oeding, P., G ov A & Myklestad B.* Immunochemical studies on antigen preparations from *Staphylococcus au eus* 2. Precipitating and erythrocyte-sensitizing properties of protein A (antigen A) and related substances. Acta path. microbiol. scand. *62* 117–127 1964
37 *Porath, J., Axén R & Ernback S* Chemical coupling of proteins to agarose. N ture (Lond.) *215* 1491–1492, 1967
38. *R ml, G & Nisonoff A.* Crystallization of fragment F b of human IgG myeloma proteins. Biochem. Biophys. Res. Commun. *31* 914–918 1968.
39. *Sabatini D D., Bensch, K & Barrnett R J*

Cytochemistry and electron microscopy The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. J Cell. Biol *17* 19–58 1963
40 *Saussure V A de & Dandliker W B.* Ultracentrifuge studies on the effects on thiocyanate ion on antigen-antibody systems. Immunochemistry *6* 77–83 1969.
41 *Scott G., Avrameas S & Bernhard W.*. Étude au microscope électronique de la formation d'anticorps à l'aide de la phosphatase alcaline utilisée comme antigène. C. R. Acad. Sci. Ser D *266* 746–748, 1968.
42 *Sharabadi M S & Yamamoto T.*. A method for staining intracellular antigens in thin sections with ferritin-labelled antibody J Cell. Biol. *50* 246–250 1971
43 *Singer S J.*. Preparation of an electron-dense antibody conjugate Nature (Lond.) *183* 1523–1524 1959
44 *Singer S J.*. The properties of proteins in nonaqueous solvents. Adv Protein Chem. *17* 1–68, 1962.
45. *Spurr A R.*. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. *26* 31–43 1969.
46. *Wagner M & Wagner B.*: Immunelektronenmikroskopischer Nachweis von Zellwandantigenen bei Streptokokken. I. Vergleichende Darstellung des M Proteins von *Streptococcus pyogenes* mit ferritin- peroxydase- und ferrocenmarkierten Antikörpern. Zbl. Bakt. Hyg I Abt. Orig. A *222* 468–483 1972.

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THE ULTRASTRUCTURE OF CULTIVABLE TREPONEMES

3 *Treponema genitalis*

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Hougen, K. Hovind. The ultrastructure of cultivable treponemes. 3. *Treponema genitalis*. Acta path microbiol. scand. Sect. B, 83 91–99 1975

Cells of *Treponema genitalis* were studied in the electron microscope by means of negative staining and ultrathin sectioning techniques. All cells were covered by regularly structured surface layer. This layer appeared to consist of pairs of thin fibrils attached to an amorphous layer. This amorphous layer in turn is probably identical with the exterior part of the outer membrane of the organism. The pairs of thin fibrils located on this surface were interconnected by polygons. The treponemes were regularly coiled and had somewhat tapered ends with 2–4 flagella inserted at each end. The two bundles of flagella were entwined around the cytoplasmic body of the cell and interdigitated in the middle region of the organism. Treatment of cells of *T. genitalis* with *Myxobacter* AL-1 protease 1 or with deoxycholate did not reveal intra cytoplasmic tubules. This is in contrast to the results obtained with similar treatments of all other strains of species of *Treponema* hitherto examined.

Key words *Treponema genitalis* ultrastructure

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This paper presents the third part of a comparative study on the ultrastructure of different species of cultivable treponemes, the first two parts of which were completed recently (8, 9) The study was undertaken in order to determine the morphological characteristics of these organisms in the hope that any differences observed between species and /or individual strains could be of taxonomic significance for the classification of organisms of the genus *Treponema*. In the course of this comparative study it was found that the ultrastructure of cells of *Treponema genitalis* differed from that of cells of the other species of treponemes. It was therefore decided to report the results of the investigations on this spirochete separately This is the purpose of the present communication.

MATERIAL AND METHODS

The strain of *Treponema genitalis* was obtained from Professor *P H Hardy* Department of Microbiology Johns Hopkins University School of Medicine, Baltimore, Maryland USA. Originally Professor *Hardy* received this strain from Dr *G R Cannefax* Center for Disease Control, Atlanta, Georgia, USA, labelled VDRL-2 (personal communication) It has not been possible to trace the actual origin of the strain further

The cells were grown at 36 C in an atmosphere of 95 per cent N and 5 per cent CO In medium consisting of 45 per cent spirolate broth (Baltimore

Biological Laboratories) 45 per cent brain heart infusion broth (Difco) and 10 per cent inactivated normal rabbit serum. After 5–6 days of growth, cells were harvested by centrifugation as stated earlier (8)

Treatment with the detergents Teepol and sodium deoxycholate and the enzyme *Myxobacter* AL-1 protease 1 (AL 1 enzyme) as well as the procedures for negative staining sectioning and electron microscopy have all been described previously (8)

For this work approximately 400 recordings were studied.

RESULTS

Observations on Negatively Stained Material

Treponema genitalis was a slender helical microorganism 4–11 μm long (Fig 1) The wavelength of the cells was quite regular and was approximately 1 1 μm. The amplitudes along individual cells were somewhat irregular but a mean value of about 0 15 μm was estimated. The tips of the organisms were slightly tapered (Figs. 1 2) The cells were covered by a regularly structured surface layer (RS) (Fig 2) The total width of the treponeme including this RS layer was 0.24 μm. while the width of the cytoplasmic body alone was 0 14 μm.

The number of flagella inserted at the ends of the cells varied 65 per cent of the ends of the cells examined had 3 flagella inserted (Fig. 2) while 25 per cent of the ends had 4 and in the remaining 10 per cent only 2 flagella were present. The insertion points of the flagella were often observed aligned in a row with a distance of about 76 nm between two adjacent insertion points (Fig 2) The distance from the outermost point of insertion to the cytoplasmic membrane at the end of the cell was 90–100 nm. The two bundles of flagella were twined together with the cytoplasmic body and individual flagella of each bundle interdigitated in the middle region of the cells

The organisms retained their helical shape after treatment with 0.2 per cent Teepol for 30 seconds. The RS layer still covered the cells and was apparently not affected by the treatment (Fig 3 A 30 second Teepol treatment liberated very few flagella from the cells, while almost all flagella were freed from cells treated with Teepol for more than 1 minute. The cytoplasm of cells treated with Teepol generally showed a mottled appearance (Fig. 3)

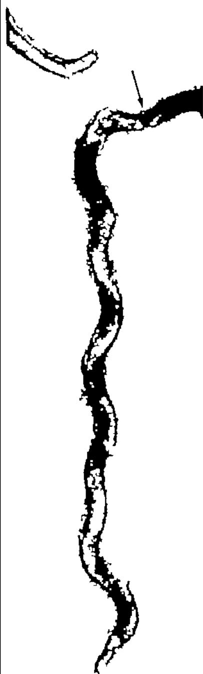
Very few flagella were liberated from cells treated with 1 per cent sodium deoxycholate for 30 seconds or 1 minute (Fig 4) However this treatment sometimes damaged the regular pattern of the RS layer Thin fibrils with a diameter of about 2 nm were visible in such preparations and the fibrils appeared to originate from the isolated flakes of the RS layer which were removed from the surface of cells as a result of the treatment (Fig 5)

Treatment with AL-1 enzyme for 30 seconds had little effect on the shape of *T genitalis* cells apart from a moderate swelling of the RS layer If however the treatment of cells with AL-1 enzyme was extended to 1-2 minutes, the regular wavy outline of the cells was affected. Flagella were inserted in deformed cytoplasmic remnants and were held together in the original group of 2 to 4 (Figs. 6, 7) The flagella emerging from the cytoplasmic remnants were enveloped in fragments of the RS layer (Figs. 6, 7) The pattern of the RS layer was frequently somewhat damaged. Thin fibrils with a diameter of about 2 nm were often visible in isolated flakes of the RS layer and patches without visible substructure were also observed (Fig 7)

All figures show material obtained from cultures of *T genitalis* The material on Figs. 1 10 negatively stained with 1 per cent ammonium molybdate pH 7 The bar on each micrograph represents 100 nm, unless otherwise stated.

Fig 1 A cell with an irregular amplitude and slightly tapered ends. This cell is one of a pair in a dividing cell. The site of division is seen (arrow) × 30,000.

Fig 2 Cell covered by a regularly structured surface layer (RS) The end is slightly tapered and has 3 flagella (F) The insertion points (IP) are aligned in a row × 160 000.



IP

F

RS

2



Fig 3 Cell treated with 0.2 per cent Teepol for 30 seconds. The RS layer is unaffected by the treatment and the cell has retained its helical shape. The flagella (F) are attached to the cell. Note the mottled appearance of the cell cytoplasm. × 90,000

Fig 4 This organism was treated with 1 per cent sodium deoxycholate for 1 minute. The flagella are still inserted in the cytoplasm (arrows). × 160,000.

Fig 5 Isolated flakes of the RS layer of cells treated with sodium deoxycholate for 30 seconds. The thin fibrils of the RS layer are visible. × 160,000

The substructure of flagella and their insertion organelles was studied on flagella liberated by treatment of cells with Teepol. For the purpose of the present study the insertion part of the flagellum will be considered to be the proximal part. At this end of the flagellum a hook with a honey-combed substructure and a diameter of 15–16 nm was



Figs. 6–7 Remnants of cells treated with *Myxobacter* AL 1 protease 1 for 2 minutes (Fig. 6) and 4 minutes (Fig. 7). The flagella (F) are inserted in remnants of cytoplasm. The flagellar bundles are enveloped by the RS layers. Note patches (P) where no substructure is visible in the RS layer × 90,000

present and this hook connected the shaft to the basal knob (Figs. 8 9). A narrow collar about 16 nm long and 9.5 nm wide connected the hook to the basal knob (Figs. 8, 9). The shape of the basal knob was usually obscured by adhering pieces of membrane but appeared on a few micrographs to consist of two thin discs or rings in close apposition to each other (Fig. 9). The diameter of the discs was about 30 nm. The shaft of the flagellum was covered by a sheath which frequently did not extend along the entire length of the flagellum but left the distal end unsheathed. The diameter of the sheathed flagellum was 18 nm and that of the core about 12 nm.

A high concentration of flakes of the RS layer was obtained simultaneously with a high concentration of free flagella after differential centrifugation of cells treated in suspension with Teepol. A more purified RS layer preparation was readily obtained by high speed centrifugation of the supernatant from a cell suspension which had been heated to 56 C for 1 hour. The cells were subsequently removed by low speed centrifugation.

The substructural pattern of the RS layer was the same irrespective of the type of isolation procedure used (Figs. 8, 9 10). The pattern was difficult to resolve as most of the flakes on the micrographs showed moiré patterns, but the pattern appeared to consist of irregular holes or pits in which negative stain had accumulated. The overall diameter of these pits was about 5 nm, and they were situated about 4 nm apart. Thin fibrils with a diameter of about 2 nm which frequently occurred in pairs were visible where the regular pattern was damaged (Fig. 10).



Figs 8–9 Flagella and flakes of the RS layer isolated from cells treated in suspension with Teepol. Knobs (B), collars (C) and hooks (H) are seen on the flagella. Moiré patterns resulting from two or more superimposed layers are visible on the flakes. × 160,000.

Fig 10 Flakes of RS layers isolated from cells heated to 56 C for 1 hour. Thin fibrils are visible (arrows) as well as a patch (P) where no substructure is seen. S indicates an area showing hexagonal symmetry of packing of subunits. × 160,000

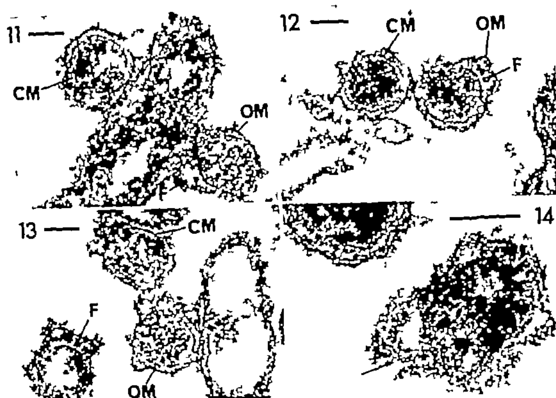
Amorphous patches with no detectable substructure were observed in between the regular substructural patterns (Figs. 6, 7, 10).

Observations on Sectioned Material

The diameter of sectioned cells of *T. genitalis* was 0.18 μm. The cells were covered by a three-layered outer membrane about 9.5 nm wide. This outer membrane appeared to be asymmetric with the outer dense layer somewhat wider and more electron dense than the inner (Figs. 12, 13). The three-layered cytoplasmic membrane bordering the cytoplasmic bodies of the cells also appeared to be asymmetric with the outer leaflet both wider and more electron dense than the inner (Figs. 11, 12). A narrow electron dense layer about 6 nm wide, was visible in a few cells which had a symmetric cytoplasmic membrane. This layer was observed just outside the outer leaflet of the cytoplasmic membrane from which it was separated by a narrow electron lucent gap.

In transversely sectioned cells the flagella were observed between the outer cell membrane and the cytoplasmic membrane (Figs. 12, 13). The diameter of cross-cut flagella was about 20 nm.

The majority of cells had a rather dense cytoplasm with evenly distributed ribosomes, but a few cells contained a more electron lucent cytoplasm with fewer ribosomes (Fig. 13).



Figs. 11–14 show sectioned cells of *T. genitalis*.

Figs. 11–13. The outer membrane (OM) appears to be asymmetric with the outer dark leaflet more electron dense than the inner. The cytoplasmic membrane (CM) is also asymmetric with the outer dark layer broader than the inner. The flagella (F) are situated between the outer membrane and the cytoplasmic membrane. A dense and uniform packing of ribosomes generally results in an electron dense appearance of the cell cytoplasm, but some cells with electron lucent cytoplasm are also seen (Fig. 13). × 90,000.

Fig. 14. Thin fibrils are visible in what is believed to represent the RS layer in an obliquely sectioned cell (arrow). × 175,000.

Thin fibrils were visible in the outer membrane of obliquely sectioned cells (Fig. 14). These fibrils were about 2.5 nm thick, and the spacing between each was generally about 5 nm.

DISCUSSION

The length and diameter of *T. genitalis* cells are of the same magnitude as reported for other species of treponemes (8, 9, 11, 12, 14, 16, 18, 19). The exterior diameter of negatively stained material (0.24 µm) may be too large because the organisms are somewhat flattened on the grids during the drying process. Probably the most correct value is obtained from the embedded material (i.e. 0.18 µm).

The dimensions of the flagella and their insertion organelles are all in complete agreement with reports on flagella of other species of *Treponema* (2, 7, 8, 9, 10, 11, 12, 13) as well as with flagella from different species of *Spirochaetae* (5, 6, 15). The structures and dimensions of the flagella insertion organelles are similar to the corresponding structures on flagella isolated from gram-positive organisms (1, 4). Although the structure of the basal knob cannot be seen clearly in the micrographs, as cytoplasm or membranous

debris usually obscures the structures, they give the impression that the basal-body complex consists of a disc or pair of discs mounted on a rod which again is connected to the hook. It is interesting to note that the basal knob on flagella isolated from *Treponema* spp. resembles that which *De Pamphilis* & *Adler* observed on flagella isolated from *Bacillus* spp. (4) whereas the basal knob on flagella isolated from *Leptospira* spp (3 17) resembles that isolated from *Escherichia coli* (4) In spite of the fact that both treponemes and leptospires are surrounded by the cytoplasmic membrane mucopeptide-outer membrane complex of the type present in gram negative bacteria.

Treatment of *T genitalis* cells with sodium deoxycholate or AL-1 enzyme did not reveal any cytoplasmic tubules in the bodies of the organisms, as is the case when cells of other species of *Treponema* are treated with these reagents under similar conditions (7 8, 9 10 11 12) It has been proposed that the presence or absence of cytoplasmic tubules in cells of the family included in the *Treponemataceae* could be of taxonomic significance (7) This point will be discussed further in a later publication.

The substructural pattern of the regularly structured cell wall surface layer is difficult to interpret, since most available micrographs show moiré patterns from two or more superimposed layers of the RS flakes present on the grid. The ultrastructure of a seemingly undamaged layer appears to consist of hexagonally packed irregular holes or pits in which the negative stain has accumulated. Thin fibrils frequently occurring in pairs become visible when the regularity of this pattern is disturbed Flakes of the surface layer which are still more damaged show patches with no detectable substructure. The arrangement of the morphological components of the RS layer of *T genitalis* resembles that of the RS layer on cells of *Treponema refringens* (8) For both organisms an amorphous layer is found in close apposition to the outer leaflet of the outer membrane of the cell. This layer may actually constitute the exterior part of the outer membrane. Pairs of long thin fibrils about 5 nm apart are situated on the surface of this amorphous layer

Polygons of a size big enough to interconnect the fibrillar pairs are situated with a regular spacing on top of these fibrils. Preliminary results of optical diffraction analysis on micrographs obtained from flakes of RS layers indicate that for cells of *T genitalis* the polygons are in fact hexagons.

I wish to express my gratitude to Dr *H Agn Nielsen* and civ.ing. *A Birch-Andersen* for their help and encouragement during this study The excellent assistance in electron microscopic and photographic work of Mr *F Lassen* Mrs *H Race* Mrs *J Berg* and Miss *A G Osterpard* is greatly appreciated.

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REFERENCES

1 *Abram D., Vatter A E & Koffler H* Attachment and structural features of flagella of certain bacilli. J Bact. *91* 2045-2068, 1966.
2. *Bharier M A Eiserling F A & Rittenberg S C* Electron microscopic observations on the structure of *Treponema zuelzerae* and its axial filaments. J Bact. *105* 413-421 1971
3 *Birch-Andersen A Hovind Hougen K & Borg-Petersen C* Electron microscopy of *Leptospira*. I *Leptospira* strain Pomona. Acta path. microbiol. scand Sect. B, *81* 665-676, 1973
4 *De Pamphilis M L & Adler J* Fine structure and isolation of the hook-basal body complex of flagella from *Escherichia coli* and *Bacillus subtilis*. J Bact *105* 384 393 1971.
5 *Hespell R. B & Canale-Parola E. Spirochaeta litoralis* Sp n a strictly anaerobic marine spirochete. Arch Mikrobiol. *74* 1 18, 1970.
6. *Holt S C & Canale-Parola E.* Fine structure of *Spirochaeta stenostrepta* free-living, anaerobic spirochete J Bact 96 822 835, 1968
7 *Hovind Hougen K* Further observations on the ultrastructure of *Treponema pallidum* Nichols. Acta path. microbiol scand. Sect. B, *80* 297 304 1972
8 *Hovind Hougen K* The ultrastructure of cultivable treponemes I *Treponema phagedenis T ponema vincentii* and *Treponema re-*

fringens. Acta path. microbiol. scand. Sect. B, 82 329–344 1974

9. *Hovind Hougen K.*. The ultrastructure of cultivable treponemes. II *Treponema calligyrum Treponema minutum* and *Treponema microdentium*. Acta path. microbiol scand. Sect. B, 82 495–507 1974

10. *Hovind Hougen K. & Birch Andersen A.*. Electron microscopy of endoflagella and microtubules in *Treponema* Reiter Acta path. microbiol. scand. Sect. B, 79 37–50 1971

11. *Hovind Hougen, K., Birch-Andersen A & Jensen, H J Skovgaard* Electron microscopy of *Treponema cuniculi* Acta path. microbiol. scand. Sect. B 81: 15–26, 1973.

12. *Hovind Hougen, K., Jensen H J Skovgaard Birch-Andersen A & Blom J.:* Electron microscopy of *Treponema pertenue* Ultrastructure of organisms and their location in skin lesions and lymph nodes of experimentally infected hamsters To be published in Acta path. microbiol. scand

13. *Jackson S & Black S H* Ultrastructure of *Treponema pallidum* Nichols following lysis by physical and chemical methods. II Axial filaments. Arch. Mikrobiol. 76 325–340, 1971

14. *Jepsen O B Hovind Hougen K. & Birch Andersen, A.*. Electron microscopy of *Treponema pallidum* Nichols. Acta path. microbiol. scand. 74 241–258, 1968.

15. *Joseph R & Canale-Parola, E.*. Axial fibrils of anaerobic spirochetes Ultrastructure and chemical characteristics. Arch. Mikrobiol. 81 146–168 1972.

16. *Moreau M & Ginolini J.*. Étude au microscope électronique de quatre espèces de tréponèmes anaérobiques d'origine génitale. Ann. Inst. Pasteur 90 728–737 1956.

17. *Nauman R K., Holt S C. & Cox C D.:* Purification, ultrastructure and composition of axial filaments from *Leptospira*. J Bact. 98 264–280, 1969.

18. *Pillot J & Ryter A.:* Structure des spirochètes. I Étude des genres *Treponema Borrelia* et *Leptospira* au microscope électronique. Ann. Inst. Pasteur 108 791–804 1965

19. *Socransky S S., Listgarten, M., Hubersak C Cotmore J & Clark A.:* Morphological and biochemical differentiation of three types of small oral spirochetes. J Bact. 98 878–882, 1969

Acta path. microbiol. scand. Sect. B, 83 100-102, 1975

INFLUENCE OF THERAPEUTIC CONCENTRATIONS OF PHENYLBUTAZONE ON GRANULOCYTE FUNCTION

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Solberg, C. O. Influence of therapeutic concentrations of phenylbutazone on granulocyte function. Acta path. microbiol. scand. Sect. B, 83 100-102 1975

The influence of therapeutic concentrations of phenylbutazone on human granulocyte function has been examined using a method which facilitates a precise *in vitro* evaluation of the phagocytic and bactericidal activities of polymorphonuclear leucocytes. Phenylbutazone caused a marked reduction in intracellular killing of bacteria by the granulocytes. Whether this inhibition of granulocyte function also takes place *in vivo* resulting in enhanced susceptibility to infection, remains unknown.

Key words Granulocyte function phenylbutazone.

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In recent years, several disease syndromes characterized by chronic bacterial or fungal infections have been related to inherited defects in either phagocytosis or intracellular killing of bacteria by neutrophil granulocytes (for review see 2, 5 7) Evidence is now accumulating that also commonly used therapeutic agents might influence granulocyte function (for review see 5 7) In high concentrations, phenylbutazone, a clinically useful anti-inflammatory drug, have been found to reduce both phagocytic and bactericidal activities of the granulocytes (8) In the present study using a sensitive method which facilitates a precise *in vitro* evaluation of both phagocytic and bactericidal activities of the granulocytes, we demonstrate that also therapeutic concentrations of phenylbutazone significantly reduces granulocyte function.

MATERIALS AND METHODS

The phagocytic and bactericidal activities of the granulocytes were determined as previously described (6, 9) 0.5 ml suspension of human leucocytes containing 5×10^6 neutrophils, 0.4 ml 25 per cent pooled human serum as opsonin and 0.1 ml suspension of *Staphylococcus aureus* "Oxford" Heatley strain (12–18 × 10 colony forming units per ml) as test organism were added to 12 × 75 mm plastic tubes. This provided about 3 bacteria per neutrophil granulocyte and final concentration of 10 per cent serum. In the tests with phenylbutazone 20 40, 80 and 160 μg of the drug (manufactured by Geigy Basel, Switzerland and used at a concentration of 200 mg per ml solution) were added to the leucocyte suspensions and diluted serum immediately before mixing with the bacteria suspensions. In the control tests, only phenylbutazone was not included. The tests were incubated at 37 C and samples were removed at prescribed intervals for the determinations of the total number of viable bacteria and the number of viable intracellular bacteria as previously described (6) The bactericidal capacity of the granulocytes is propor-

ional to the total number of bacteria killed and inversely proportional to the total number of viable bacteria or the number of viable intracellular bacteria (6, 9) The number of bacteria phagocytized equals the number of viable intracellular bacteria plus the number of bacteria killed (6, 9)

RESULTS

In the tests containing no phenylbutazone a marked reduction in total viable bacteria was observed and very few intracellular bacteria remained viable after incubation for 2 hours (Figs. 1 and 2) indicating rapid phagocytosis and intracellular killing of bacteria. In contrast, the reduction in total viable bacteria in the tests containing phenylbutazone was significantly less pronounced and the number of viable intracellular bacteria increased with increasing phenylbutazone concentrations (Figs. 1 and 2) indicating normal or close to normal phagocytosis but significantly reduced intracellular killing of bacteria. Less than 20 μg phenylbutazone per ml did not influence granulocyte function.

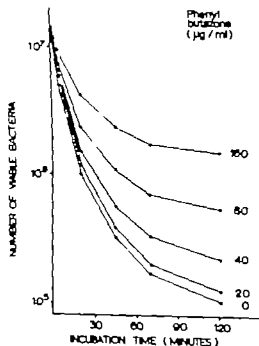


Fig. 1 Total number of viable bacteria during incubation of leucocyte-bacteria suspensions with and without phenylbutazone (mean of five experiments)

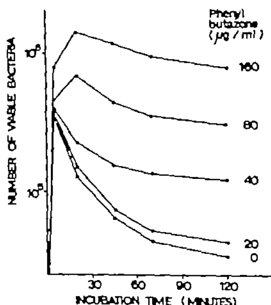


Fig. 2 Number of viable intracellular bacteria during incubation of leucocyte-bacteria suspensions with and without phenylbutazone (mean of five experiments)

DISCUSSION

In previous studies, it has been demonstrated that high concentrations of phenylbutazone effectively inhibit the bactericidal activity of the granulocytes (8, 10) Together the granulocyte enzyme, myeloperoxidase, a halide and hydrogen peroxide form a strong antimicrobial system and the impaired bactericidal activity of the granulocytes in the presence of phenylbutazone seems to be due to decreased glucose-6-phosphate dehydrogenase and hexose monophosphate shunt activity resulting in poor hydrogen peroxide formation (8, 10) Whether this inhibition of granulocyte enzyme activity also takes place *in vivo* resulting in enhanced susceptibility to infection, remains unknown. However we have treated two young patients with apparently normal antimicrobial host defence mechanisms who developed septicaemia while on long-term phenylbutazone therapy Furthermore phenylbutazone is rapidly absorbed from the gastro-intestinal tract and following therapeutic doses, plasma levels of 50–120 μg per ml are usually reached (1 3 4) In the

present study drug concentrations of this magnitude significantly reduced the intra cellular killing of bacteria by the granulocytes. Also the biotransformation of phenylbutazone is slow averaging about 20 per cent per day and plasma levels of 20–40 μg per ml may be found as long as seven days after therapy has been discontinued (3 4) Finally as demon strated in previous studies, the diffusion of the drug into the granulocytes is very rapid (6, 8, 9) On the other hand, the protein binding of phenylbutazone is high (3) and this may reduce the diffusion of the drug across the granulocyte membrane. Accord ingly to determine whether phenylbutazone therapy in man may reduce antimicrobial host defence, extensive clinical observations seem indicated.

REFERENCES

1 *Burck E. Fearnley M E., Meanock I & Patley H* Phenylbutazone therapy Relation between the toxic and therapeutic effects and the blood level. Lancet *1* 225–228, 1954
2. Disorders of neutrophil function. Lancet *1* 438–440, 1974
3 *Goodman L. S & Gilman A.* The pharma cological basis of therapeutics. Fourth edition The Macmillan Company London & Toro 1970 pp. 335–337
4 *Heidberg, E. F., Andreasen P B. & Ravi L.* Plasma half-life of phenylbutazone in p tients with impaired liver function. Cl Pharmac. Therap. *15* 171–177 1974
5 *Quie P G* Disorders of phagocyte functio Year Book Medical Publishers, Inc., Chicag 1972.
6. *Solberg C O.* Enhanced susceptibility to i fection. A new method for the evaluation neutrophil granulocyte functions. Acta pat microbiol. scand. Sect. B, *80* 10–18, 1972.
7 *Solberg C O* Enhanced susceptibility to i fection. Disorders of phagocyte function. Spe trum International *17* 41–47 1974
8 *Solberg C O.* Influence of phenylbutazo on the phagocytic and bactericidal activities neutrophil granulocytes. Acta path. microbi scand. Sect. B, *82* 258–262, 1974.
9 *Solberg C O* Protection of phagocyte bacteria against antibiotics. A new method f the evaluation of neutrophil granulocyte fun tions. Acta med. scand *191* 383–387 1972.
10 *Strauss R R., Besney B P & Sbarra, A. J* Effect of phenylbutazone on phagocytosis an intracellular killing by guinea pig polymorph nuclear leucocytes. J Bacteriol. 96 19– 1990, 1968.

Acta path. microbiol. scand. Sect. B, 83 105–115 1975

CORRELATION BETWEEN TWITCHING MOTILITY AND POSSESSION OF POLAR FIMBRIAE IN *ACINETOBACTER CALCOACETICUS*

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Henrichsen, J. & Blom, J. Correlation between twitching motility and possession of polar fimbriae in *Acinetobacter calcoaceticus*. Acta path. microbiol. scand. Sect. B 83 105–115 1975.

Negatively stained preparations of 16 strains of *Acinetobacter calcoaceticus* were examined in the electron microscope. Five of the strains did and 11 of the strains did not exhibit twitching motility. Two of the non-twitching strains were substrains of twitching wild-type strains. All twitching strains were found to possess fimbriae with a diameter of approximately 50 Å. These fimbriae were shown to be of polar origin except in one strain where, for technical reasons, the origin could not be determined with certainty. Polar fimbriae could not be demonstrated in any of the strains that did not exhibit twitching motility. The demonstration of polar fimbriae was only regularly possible during the exponential growth phase, presumably because the fimbriae are shed by the bacteria during later growth phases. During the study methods were developed for securing exponentially growing bacteria in concentrations suited for the preparation of negatively stained cells for electron microscopy. Peritrichously arranged fimbriae with a diameter of approximately 30 Å were demonstrated in nearly all strains studied, especially on cells in late growth phases. The occurrence of this kind of fimbriae was therefore not correlated with the occurrence of twitching motility.

Key words: *Acinetobacter calcoaceticus*; twitching motility; polar fimbriae.

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Bacterial fimbriae (9)—or pili (5)—have been defined as "any morphologically distinct filamentous appendage of bacterial cells other than flagella" by Brinton (6). Duguid (7) describes fimbriae as "the non-flagellar filamentous appendages of bacteria" and he further specifies these structures as being unrelated to motility and demonstrable with the electron microscope only. However, the results reported in this paper demonstrate that some fimbriae are related to motility although it is motility of the special kind called twitching.

Twitching motility is one out of the six different types of bacterial surface translocation known at present, viz. swarming, swimming, gliding, twitching, sliding and darting (13). The mechanism of twitching—as well as that of gliding—is virtually unknown, although a

correlation between fimbriation and twitching motility in colony variants of three species of *Moraxella* (16) has been demonstrated previously

In order to investigate whether the same correlation exists in other bacterial species exhibiting twitching motility we have examined a number of strains of different bacterial species in the electron microscope. In the present paper we report the results achieved with *Acinetobacter calcoaceticus* strains. During the course of this investigation it was noted that fimbriation in this species varied with the growth phase of the bacteria. Accordingly fimbriation in relation to growth phase was also made a subject of this study

In a subsequent paper the results of electron microscopical examinations of other bacterial species known to be able to show twitching motility will be reported (15)

MATERIALS AND METHODS

The Bacterial Strains studied are listed in Table 1

Agar Plate Microscopy was carried out as described previously (13) and was always performed prior to the preparation of specimens for electron microscopy in order to check whether or not the bacteria selected were exhibiting twitching motility

Media Cytophaga agar plates were used in the following modifications *No. 62* was made of 0.05 per cent tryptone (Difco) 0.05 per cent yeast extract (Difco) and 1 0 per cent agar (Difco) with a final pH adjusted to 7.0. *No. 70* was made as no. 62, but with a tryptone concentration of 0.5 per cent. In this study no. 70 was also used with sodium-taurocholate added to a concentration of 0.05 per cent for the isolation of sliding variants of twitching strains, a spreading by means of twitching motility is suppressed on this medium, whereas sliding is not (*Henrichsen* unpublished observation) Finally 62 was also used as liquid culture medium without agar added.

Incubation Culture plates were incubated at 30° C (unless otherwise stated) in closed jars with a layer of water in the bottom in order to ensure a humid atmosphere

Preparation of Specimens for Electron Microscopy: As fimbriation might conceivable vary with the stage of growth of the bacteria, a number of different preparations were made for negative staining. In all cases the stain used was 1 0 per cent (w/) ammonium molybdate adjusted to



Fig 1 Strain A 135 One half of a 6 hour-old microcolony on cytophaga agar no. 62. Approximately 300 ×

pH 7 4 with NH_4OH. Formvar coated carbon reinforced copper grids were irradiated for 10 min with ultraviolet light prior to use. Throughout, care was taken to reduce manipulation of the bacteria to a minimum in order to avoid mechanical shearing of fimbriae The following preparations for negative staining were used

1 *Bacteria Grown on Solid Media*

a) *"Suspension method"* Using a platinum loop, a suitable amount of bacteria was gently removed from the outermost part of the colonies or the spreading zones—if spreading took place—and carefully suspended directly into the negative stain to give a final concentration of approximately 10^8 cells/ml. A grid was then applied film side down, on top of a drop of this suspension placed on a strip of Parafilm®. After 1 min, the grid was removed and excess liquid was sucked off by a piece of filter paper The "suspension method" gave poor results in comparison with the other methods as shown by the fact that polar fimbriae were demonstrated by this method in only one case.

b) *"Drop method"* This method was used for strains exhibiting spreading growth. After examination of the spreading zone under the light microscope in order to ensure that twitching motility actually was taking place large drop of the negative stain was placed directly on the agar surface so that the drop touched and partly covered the outer part of the spreading zone It was noted by microscopical examination that the cells from the outer part of the spreading zone after application of the drop floated on th drop surface Immediately a grid was placed film side down on the liquid surface After 5 to 10 seconds, the grid was removed and excess liquid ucked off by filter paper For comparativ purposes, corresponding preparations were also made from the central part of the growth area, and from somewhere between

the centre and the edge of the spreading zone after having carefully removed the bacteria of the spreading zone from the agar surface.

c) *"Wash off method"* Freshly poured plates were dried for 30 min at 35 C and then floated with a suitably diluted liquid culture (medium no. 62) of bacteria so as to give approximately 1000 colonies per plate. Excess of the fluid culture was sucked off by a Pasteur pipette and the plates were left on the table to dry for 5 min before incubation. After varying times of incubation—depending on the growth rate of the strain in question—when the colonies were of suitable size and microscopical examination showed twitching motility of all cells in the colony (Fig. 1) the surface growth on two or three plates was gently washed off with 1 ml of the negative stain using a Pasteur pipette. A grid was then placed on top of one drop of the resulting bacterial suspension and after about 1 min it was removed and excess liquid sucked off as before. The method was also used for washing off young colonies of non-spreading strains and in a number of instances it was even used for washing off bacteria of 20 hour-old colonies. This was found to be satisfactory partly because it could be seen that only the outer part of the colonies was washed off and partly because the electron micrographs showed that the bacteria on the grids were definite rods indicating, according to *Bergmann et al.* (1) that they were in the exponential growth phase.

2. Bacteria Grown in Liquid Medium

0.5 ml of a 9 hour-old liquid culture was used to inoculate 5 ml of liquid medium no. 62. This was then incubated for 4 hours at 35 C and slowly shaken. Preliminary growth experiments had shown that bacteria grown in this way are in the transition phase between late logarithmic growth phase and early stationary phase. Shorter incubation periods did not give bacterial densities sufficient for electron microscopy and centrifugation and resuspension were judged to be too damaging to fimbriae possibly present. A grid was applied on top of a drop of the 4 hour-old culture placed on a strip of parafilm. After 30–60 seconds the grid was removed and excess liquid sucked off. Finally the grid was placed on top of a drop of the negative stain for another minute removed and excess stain sucked off.

Electron Microscopy in all cases was carried out at 60 kV on a Philips EM 200 electron microscope at primary magnifications of 9000 × Negatives were obtained on Kodak Fine Grain Release Positive Film Type 5302 and suitable fields were photographically enlarged as desired.

Measurements By the aid of a measuring lens (7×) graded into tenths of millimeters, the diameters of fimbriae were measured directly on photographs of the negatively stained preparations at a final magnification of 90,000 × Measurements were always performed on isolated fimbriae.

Nomenclature Two kinds of fimbriae were observed, viz. fimbriae with a diameter of approximately 50 A and those with a diameter of approximately 30 A. They are hereafter in this paper referred to as thick and thin fimbriae, respectively

RESULTS

Variation of Fimbriation with Bacterial Growth Phases

Four strains exhibiting twitching motility (A 16, A 135 ATCC 17905 and AB 156) were studied extensively for the presence of fimbriae under different conditions of growth using all the described methods of preparation for negative staining In addition, fimbriae were repeatedly sought for under different conditions in the non-twitching strain A 79 Fig 2 shows part of the polar region of a cell of this strain negatively stained from a 4 hour-old liquid culture. The cell surface is convoluted and fimbriae are not seen. In fact, fimbriae were not found in any of the preparations made of this strain either on the coccoid cells of the stationary phase or on the rods of the exponential phase. A small bundle of the appearance of thin fimbriae was noticed on one picture taken from a preparation made from a liquid culture. However this bundle might just as well be interpreted as slime threads which are often observed in preparations of this mucoid strain. Thick fimbriae, found on all the twitching strains studied were never encountered on cells of strain A 79

The four twitching strains all had polarly arranged thick fimbriae on the majority of the cells of the exponential growth phase These fimbriae were usually relatively straight and often very long 10–15 µm. They had an even diameter along their entire length and in most cases were without discernible substructure. Figs. 3–5 show the variation in appearance of strain A 16 at different stages of growth. The cells vary from long rods with a tuft of polarly localized thick fimbriae in the exponential phase (Fig 5) to small coc







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coccoid cells with occasional thin fimbriae in the stationary phase (Fig. 3) The cell shown in Fig. 4 represents a stage somewhere in between (the preparation stemming from the area just central to the outer spreading zone) being a little larger and somewhat more rod-like than the one shown in Fig 3 and covered by a fur of thin and rather curly fimbriae. Only few thick fimbriae the origin of which cannot be determined precisely are seen. The same pattern of variation is also evident in strains ATCC 17905 AB 156 and A 135 the coccoid cells are covered with thin fimbriae and only few thick fimbriae are found (Figs. 6–8, 10, 11 and 14) whereas the thick fimbriae of polar origin are the only ones seen on the long rods of the exponential growth phase (Figs. 9 12, 13 and 15)

Fimbriation in Twitching and in Non-twitching Strains

A total of 16 strains were examined three of these were substrains selected from the original wild-type strains two were non twitching substrains of twitching wild-type strains and one was a non-spreading substrain of a sliding wild-type strain. The results are listed in Table 1 Five of the strains exhibited typical twitching motility including the production of spreading zones on medium no. 62. All these strains were shown to possess thick fimbriae. In the four strains already described, the origin of the fimbriae was definitely polar In the fifth strain, H 127R, the origin could not be decisively determined, although a polar localization appears likely also in this case (Fig 17) Strain H 127M a non-twitching substrain of strain H 127R, possibly had few thin fimbriae but thick fimbriae were never seen (Fig 16) however the evaluation of the photographs was difficult due to the substantial amount of slime produced by this strain. In a substrain of the twitching strain A 16, strain A 16s, which does not exhibit twitching motility on medium no. 62 but spreads by sliding if sodium-taurocholate is included in the medium, only thin fimbriae could be observed even on the long rods of the exponential growth phase.

A few thick fimbriae were observed in preparations of strain BD-4 (Fig. 19) This strain however gives rise to the formation of quite broad spreading zones due to sliding on medium no. 62 (Fig. 23 in ref 13) and this might conceivably prevent the demonstration of an existing potential for twitching motility Strain BD-4N a non-spreading substrain of strain BD-4 did not possess thick fimbriae (Fig. 18) but thin fimbriae were found on both BD-4 and BD-4N as well as on other non-twitching and twitching strains as entered in the table. Figs. 18 and 19 are taken from grids prepared by the "suspension method" but the "drop" and "wash off methods" gave similar preparations.

The rest of the strains including another sliding strain, H 142 were all devoid of twitching motility and did not possess thick fimbriae.

Four strains, P 719 P 657 Fsk 2147 and 2679 without twitching motility were examined immediately after isolation from clinical specimens in order to exclude the possibility that fresh isolates possess thick fimbriae

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Figs. 2 19 all show cells negatively stained with 1 per cent ammonium molybdate pH 7 4 All preparations are made from cytophaga agar no. 62 or its liquid equivalent. The bar on each micrograph represents 0.1 μm and all the figures are shown at final magnification of 90,000 ×

Fig 2 Strain A 79. 4 hour-old liquid culture. Part of the polar region of medium sized rod. The cell surface is highly convoluted. Fimbriae are not present.

Fig 3 Strain A 16. Polar region of coccoid rod prepared by the "suspension method" fails to reveal thick fimbriae Only few thin fimbriae with a diameter of approximately 30 Å are seen (arrow)

Fig 4 Strain A 16. Drop method" preparation from area just central to spreading zone Exactly one half of a coccoid rod with a fur of thin curly fimbriae is shown. Few thick (approx. 50 Å) fimbriae (arrow) of unknown origin.

Fig 5. Strain A 16. Drop method" preparation from outer part of spreading zone. Polar region of definet rod with tuft of thick polar fimbriae. Exactly one half of the cell is shown on the micrograph (cf with Fig. 4)

TABLE 1 *Correlation between Twitching Motility and Fimbriation in the* Acinetobacter calcoaceticus *Strains Studied*

Strain designation a)	Spreading growth b)	Twitching motility	Fimbriae approx. 50 Å c)	Fimbriae approx. 30 Å c)
A 16	+	+	+ +	+ +
A 16 d)	+	—	—	+ +
A 79	—	—	—	(+)
A 135	+	+	+ +	+ +
ATCC 17905	+	+	+ +	+ +
AB 156	+	+	+ +	+
H 127R	+	+	+ +	—
H 127M e)	—	—	—	+
BD-4	+	—	+	+
BD-4N f)	—	—	—	+
ATCC 11171	—	—	—	+
H 142	+	—	—	+
P 719 g)	—	—	—	—
P 657 g)	—	—	—	(+)
Fak 2147 g)	—	—	—	(+)
2679 g)	—	—	—	—

a) ATCC, American Type Culture Collection, Rockville Maryland, USA BD-4 strain from *E. Juni*, Department of Microbiology University of Michigan, Ann Arbor Michigan, USA all other strains listed are from Statens Seruminstitut, Copenhagen, Denmark.

b) Sliding (13) is the cause of the spreading growth of those strains listed that do spread but do not exhibit twitching motility

c) + + = many fimbriae + = few; (+) = maybe a few but difficult to evaluate with certainty due to "slime threads" Fimbriae with a diameter of approx. 50 Å appear to be polarly located and in only two strains (H 127R and BD-4) the origin of these fimbriae was not decisively determined. Fimbriae with a diameter of approx. 30 Å appear to be peritrichously arranged

d) Sliding, but non-twitching substrain of strain A 16.

e) Non-twitching and mucoid substrain of strain H 127R.

f) Non-spreading substrain of strain BD-4

g) Strains examined for spreading growth and also examined electron microscopically immediately after isolation from various clinical specimens.

which they lose during subsequent subculture. Thick fimbriae were not observed in any of these strains.

DISCUSSION

The presence of fimbriae has been shown to be correlated with twitching motility in *Moraxella nonliquefaciens* *M. bovis* and *M. kingae* (16) The fimbriae of *M. nonliquefaciens* were judged to originate from all over the surface of the cells (2) while the fimbriae of *M. bovis* "were often concentrated near the polar regions of the cells" (3) and the origin of the fimbriae of *M. kingae* apparently was not determined (10) If measured as the centre-to-centre distance between two fimbriae running in parallel the diameters of the fimbriae of these three species of *Moraxella*

Fig 6. Strain ATCC 17905 4 hour-old liquid culture Thin fimbriae on coccoid cell.

Fig 7 Strain ATCC 17905 "Suspension method" preparation from 24 hour-old plate culture Many thin fimbriae on coccoid cell.

Fig 8 Strain ATCC 17905 "Drop method" preparation from area just cent 1 to spreading zone Part of a coccoid rod with long and curly thin fimbriae some of which are arranged in bundles.

Fig 9 Strain ATCC 17905 "Wash off method" preparation from 9 hour-old microcolonies. Delicate rod with polar and subpolar thick fimbriae. Thin fimbriae are not present.

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TABLE 1 *Correlation between Twitching Motility and Fimbriation in the* Acinetobacter calcoaceticus *Strains Studied*

Strain designation[a)]	Spreading growth[b)]	Twitching motility	Fimbriae approx. 50 Å[c)]	Fimbriae approx. 30 Å[c)]
A 16	+	+	+ +	+ +
A 16[d)]	+	—	—	+ +
A 79	—	—	—	(+)
A 135	+	+	+ +	+ +
ATCC 17905	+	+	+ +	+ +
AB 156	+	+	+ +	+
H 127R	+	+	+ +	—
H 127M[e)]	—	—	—	+
BD-4	+	—	+	+
BD-4N[f)]	—	—	—	+
ATCC 11171	—	—	—	+
H 142	+	—	—	+
P 719[g)]	—	—	—	—
P 657[g)]	—	—	—	(+)
Fsk 2147[g)]	—	—	—	(+)
2679[g)]	—	—	—	—

a) ATCC, American Type Culture Collection, Rockville, Maryland USA. BD-4 strain from E. Juni, Department of Microbiology, University of Michigan, Ann Arbor Michigan, USA; all other strains listed are from Statens Seruminstitut, Copenhagen, Denmark.
b) Sliding (13) is the cause of the spreading growth of those strains listed that do spread but do not exhibit twitching motility.
c) + + = many fimbriae; + = few; (+) = maybe a few, but difficult to evaluate with certainty due to "slime threads". Fimbriae with a diameter of approx. 50 Å appear to be polarly located and in only two strains (H 127R and BD-4) the origin of these fimbriae was not decisively determined. Fimbriae with a diameter of approx. 30 Å appear to be peritrichously arranged.
d) Sliding, but non-twitching substrain of strain A 16.
e) Non-twitching and mucoid substrain of strain H 127R.
f) Non-spreading substrain of strain BD-4.
g) Strains examined for spreading growth and also examined electron microscopically immediately after isolation from various clinical specimens.

which they lose during subsequent subculture. Thick fimbriae were not observed in any of these strains.

DISCUSSION

The presence of fimbriae has been shown to be correlated with twitching motility in *Moraxella nonliquefaciens*, *M. bovis* and *M. kingae* (16). The fimbriae of *M. nonliquefaciens* were judged to originate from all over the surface of the cells (2), while the fimbriae of *M. bovis* "were often concentrated near the polar regions of the cells" (3) and the origin of the fimbriae of *M. kingae* apparently was not determined (10). If measured as the centre-to-centre distance between two fimbriae running in parallel, the diameters of the fimbriae of these three species of *Moraxella*

Fig. 6. Strain ATCC 17905. 4 hour-old liquid culture. Thin fimbriae on coccoid cell.

Fig. 7. Strain ATCC 17905. "Suspension method" preparation from 24 hour-old plate culture. Many thin fimbriae on coccoid cell.

Fig. 8. Strain ATCC 17905. "Drop method" preparation from area just central to spreading zone. Part of a coccoid rod with long and curly thin fimbriae some of which are arranged in bundles.

Fig. 9. Strain ATCC 17905. "Wash off method" preparation from 9 hour-old microcolonies. Definite rod with polar and subpolar thick fimbriae. Thin fimbriae are not present.

were found to be somewhat larger (60–85 Å) than the directly measured diameters (40–50 Å). The thick fimbriae of *A. calcoaceticus* observed in the present study had a directly measured diameter of the same order of magnitude, i.e. approximately 50 Å.

Reports on fimbriae of *Acinetobacter* are scarce and difficult to interpret as detailed information is lacking. *Thornley* (27) mentioned that many strains of her phenons 2, 3 and 4 had fimbriae, but neither the diameter nor the localization of these fimbriae was given. Besides, she did not examine her strains for twitching motility. The filamentous appendages described in the two strains of *Acinetobacter calcoaceticus* by *Ryter & Piéchaud* (24) were later proposed to be responsible for twitching motility and named "proflagella" by Piéchaud (23). These appendages were apparently peritrichously distributed and their diameter was reported to be 150 Å. Such appendages have not been met with in our study and the reason for this discrepancy is not realized. *Swanson & Goldschneider* (25) referred to filamentous material on cells of *Herellea* (now *Acinetobacter*) as fimbriae without further comments. Their pictures represent sectioned cells on which fimbriae must of necessity be difficult to see and, in our opinion, the filamentous material looks more like "slime threads" than like fimbriae.

Fig. 10. Strain AB 156. 4 hour-old liquid culture. Bundles of fimbriae apparently consisting mainly of thin fimbriae (thin arrow) are present near polar region of a coccoid cell. A few thick fimbriae (thick arrow) of underterminable origin are also seen.

Fig. 11. Strain AB 156. "Suspension method" preparation from 24 hour-old plate culture. Like in Fig. 10 both thick and thin fimbriae are present near polar region of a coccoid cell.

Fig. 12. Strain AB 156. "Wash off method" preparation from 9 hour-old microcolonies. Thick fimbriae with a polar origin on a definite rod.

Fig. 13. Strain AB 156. "Drop method" preparation from outer part of spreading zone of 18 hour-old plate culture. Thick fimbriae accumulated around polar region of a definite rod.

The results of the present study have clearly demonstrated that exponentially growing cells of strains of *A. calcoaceticus* exhibiting twitching motility possess thick polar fimbriae while such fimbriae were never found on the corresponding cells of non-twitching strains. No attempts were made to evaluate systematically either the number of fimbriae per cell or the number of fimbriated cells in a given preparation because these polar fimbriae undoubtedly were relatively loosely attached to the cells. They were usually not retrieved on cells in stationary phase and were often seen in bundles unconnected to cells. Loss of fimbriae when the culture reaches early stationary phase has also been reported for F fimbriae (6).

Thin fimbriae were found evenly distributed over the cell surface of both twitching and non-twitching bacteria, on the former typically in stationary phase. The function of these thin fimbriae is totally unknown and their occurrence is unrelated to the presence or absence of the thick fimbriae.

Neither kind of fimbriae had any correlation to gliding motion.

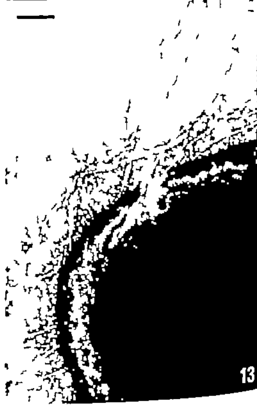
Although both *Brinton* (6) and *Duguid* (7) have proposed classification schemes for different types of fimbriae we have not attempted to identify the fimbriae of *Acinetobacter* accordingly, for the same reasons as given by *Fuerst & Hayward* (12) in their discussion of fimbriae of *Pseudomonas* spp. Present classifications of fimbriae are artificial and premature. They have already given rise to confusion (8).

Furthermore, our results have amplified the knowledge of the correlation between fimbriation and twitching motility. In strains of *A. calcoaceticus* twitching motility is correlated with the possession of *polar* fimbriae. In view of the fact that a close correlation also has been found between twitching motility and the presence of polar fimbriae with diameters of approximately 50 Å in strains of different species of *Pseudomonas* and some other gram negative rods (15), it appears likely that the fimbriae of the three above-mentioned species of *Moraxella* likewise have


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of fimbriation among strains of *N meningitidis* may be the reason why twitching motility was found in some but not all fresh isolates of this species from cerebrospinal fluid (14)

It thus appears that the correlation between twitching motility and fimbriation is in the process of being firmly established How the fimbriae are actually involved in twitching motility is not known at present.

REFERENCES

1 *Baumann P., Doudoroff M & Stanier R Y* A study of the *Moraxella* group. II Oxidative-negative species (genus *Acinetobacter*) J Bact. *95* 1520–1541 1968.

2. *Bøvre K., Bergan T & Frøholm L. O* Electron microscopical and serological characteristics associated with colony type in *Moraxella nonliquefaciens* Acta path. microbiol. scand. Sect. B *78* 765–779 1970

3 *Bøvre K & Frøholm L. O* Variation of colony morphology reflecting fimbriation in *Moraxella bovis* and two reference strains of *M nonliquefaciens*. Acta path. microbiol. scand. Sect. B *80* 629–640 1972.

4 *Bøvre K & Frøholm L. O* Competence in genetic transformation related to colony type and fimbriation in three species of *Moraxella*. Acta path. microbiol. scand. Sect. B, *80* 649–659 1972.

5 *Brinton C C Jr* Non-flagellar appendages of bacteria. Nature *183* 782–786 1959

6 *Brinton, C C Jr.* The structure, function, synthesis and genetic control of bacterial pili and a molecular model for DNA and RNA transport in gram-negative bacteria. Trans. N Y Acad. Sci. Ser II *27* 1003–1054 1965

7 *Duguid J P* The function of bacterial fimbriae. Arch. Immunol. Ther exp *16* 173–188 1968.

8. *Duguid J P & Anderson E. S* Terminology of bacterial fimbriae, or pili and their types. Nature *215* 89–90 1967

9 *Duguid J P., Smith I W Dempster G & Edmunds, P N* Non-flagellar filamentous appendages ("fimbriae") and haemagglutinating activity of *Bacterium coli* J Path. Bact. *70* 335–348 1955.

10. *Frøholm L. O & Bøvre K.* Fimbriation associated with the spreading-corroding colony type in *Moraxella kingii*. Acta path. microbiol scand. Sect. B, *80* 641–648, 1972.

11 *Frøholm L. O Jyssum, K & Bøvre K.* Electron microscopical and cultural features of *Neisseria meningitidis* competence variants. Acta path. microbiol. scand. Sect. B *81* 525–537 1973.

12. *Fuerst J A & Hayward A C.:* Surface appendages similar to fimbriae (pili) on *Pseudomonas* species. J gen. Microbiol. *58* 227 237 1969

13 *Henrichsen J.* Bacterial surface translocation. a survey and a classification. Bact. Rev *36* 478–503 1972.

14 *Henrichsen J* The occurrence of twitching motility among gram-negative bacteria. Acta path. microbiol. scand. Sect. B, *83* 171–178, 1975

15 *Henrichsen J & Blom J* Examination of fimbriation of some gram-negative rods with and without twitching and gliding motility. Acta path. microbiol. scand. Sect. B *83* 161 170 1975

16. *Henrichsen J Frøholm L. O & Bøvre K* Studies on bacterial surface translocation. 2. Correlation of twitching motility and fimbriation in colony variants of *Moraxella nonliquefaciens M bovis* and *M kingii*. Acta path microbiol. scand. Sect. B, *80* 445–452, 1972

17 *Jephcott A E., Reyn A & Birch-Andersen, A.. Neisseria gonorrhoeae* III. Demonstration of presumed appendages to cells from different colony types. Acta path. microbiol. scand. Sect B, *79* 437–439 1971

18. *Jyssum K & Lie S* Genetic factors determining competence in transformation of *Neisseria meningitidis* 1 A permanent loss of competence. Acta path microbiol. scand. *63* 306–316 1965

19 *Kellogg, D S Jr., Cohen I R., Norins, L C, Schroeter A L. & Reising, G Neisseria gonorrhoeae* II. Colonial variation and pathogenicity during 35 months in vitro. J Bact. *96* 596–605 1968.

20. *Kellogg, D S., Jr., Peacock W L Jr Deacon W E. Brown L & Pirkle C. L. Neisseria gonorrhoeae* I Virulence genetically linked to clonal variation. J Bact. *85* 1274–1279 1963

21 *Kraus S J & Glassman L H* Scanning electron microscope study of *Neisseria gonorrhoeae* Appl. Microbiol. *27* 584–592, 1974

22. *Pedersen K B. Frøholm L. O & Bøvre K* Fimbriation and colony type of *Moraxella bovis* in relation to conjunctival colonization and development of keratoconjunctivitis in cattle. Acta path. microbiol. scand. Sect. B, *80* 911–918, 1972

23 *Piéchaud M* Observations sur la mobilité des Moraxella. Abstract, IX Conference on the Taxonomy of Bacteria, Brno 1969

24 *Ryter A & Piéchaud M* Étude au microscope électronique de quelques souches de *Moraxella*. Ann. Inst. Pasteur *105* 1071–1079 1963.

5 *Swanson J & Goldschneider I.:* The serum bactericidal system: ultrastructural changes in *Neisseria meningitidis* exposed to normal rat serum. J exp. Med. *129* 51–79 1969

n. *Swanson J, Kraus S J & Gotschlich E. C.:* Studies on gonococcus infection. I. Pili and zones of adhesion, their relation to gonococcal growth patterns. J exp. Med. *134* 886–906 1971

27 *Thornley M J.:* A taxonomic study of *Acinetobacter* and related genera. J gen. Microbiol. *49* 211–257 1967

FOUR NEW *ESCHERICHIA COLI* O ANTIGENS O158, O159, O160 AND O161, AND TWO NEW H ANTIGENS, H53 AND H54

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Ørskov I., Ørskov F & Rowe, B. Four new *Escherichia coli* O antigens, O158, O159, O160 and O161 and two new H antigen, H53 and H54 Acta path. microbiol. scand. Sect. B, 83 116–120 1975

Four *Escherichia coli* strains were established as antigenic test strains for four new O groups, O158 O159 O160 and O161 The strains of O158 and O159 were isolated from cases of infantile diarrhoea in Great Britain, while the O160 and O161 strains were isolated from faecal specimens in Arabia. The O161 strain was at the same time designated as test strain of a new H antigen, H54 while a strain belonging to O group 148 was established as antigenic test strain for H53 this strain was also isolated from human faeces in Arabia.

Key words *Escherichia coli* four new O antigens two new H antigens.

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E coli strains received at the WHO Collaborative Centre for Reference and Research on Escherichia which are serologically untypable are established as test strains for new *E coli* antigens when certain conditions make it advisable (6) to do so.

MATERIAL AND METHODS

The five strains, E1020/72 E2476/72, E110/69 E223/69 and E480/68, were received at the WHO Collaborative Centre from Dr *B. Rowe* Central Publ Health Laboratory London E1020/72 and E2476/72 were found in outbreaks of enteritis in babies in Great Britain, whil the remaining three strains were isolated during study of travellers diarrhoea in British troops at Sharjah, the Arabian Gulf (7) E110/69 was isolated from faecal specimens, *mainly from soldiers* with diarrhoea but occasionally from healthy soldiers. It was also found in foods and appeared to be a common O group in the area. E223/69 was isolated from the faeces of a case of diarrhoea. E480/68 was found in the faeces of a large number of diarrhoea cases as well as in food, and appeared to be prevalent serotype in the area.

Production of antisera, methods for absorption, bacterial agglutination technique and fermentation tests were performed as described by *Kauffmann* (2) Immunoelectrophoresis was carried out according to *Scheidegger* (8) and described previously (3) Precipitation by double diffusion in gel was made with filter paper discs (5)

RESULTS AND DISCUSSION

The results of fermentation tests and other biochemical reactions are shown in Table 1 O antigen examinations in the established

TABLE 1 *Biochemical Properties of Strains Examined*

ain No.	E1020/72	E2476/72	E110/69	E223/69	E480/68
Adonitol	–	–	–	–	–
Dulcitol	+	+[a]	+	–	+
Sorbitol	+	+	+	+	+
Raffinose	+	+[a]	–	–	+
Xylose	+	+	+	+	+
Rhamnose	+	+	+	+	+
Maltose	+	–	+	+	+
Salicin	+[a]	–	+[a]	+[a]	+[a]
Inositol	–	–	–	–	–
Lactose	+	+	+	+	+
Sucrose	+[a]	+	–	–	+
Sorbose	+[a]	+[a]	–	–	–
Mannitol	+ +	+ +	+ +	+ +	+ +
Glucose	+ +	+ +	+ +	+ +	+ +
Indol	+	–	+	+	+
H_2S	–	–	–	–	–
Gelatin	–	–	–	–	–
Ammonium glucose	+	+	+	+	+
Ammonium citrate		–	–	–	–
KNO	+	+	+	+	+
Voges Proskauer	–	–	–	–	–
Methyl red	+	+	+	+	+
Urease		–	–	–	–
KCN	–	–	–	–	–
Malonate	–		–	–	–
Motility 37 C	+	(+)	+	+	+
Haemolysis	–		–	–	–
Serotype	O158 H23	O159 H20	O160 H34	O161 H54	O148 H33

+ = positive after 1 day + = positive after 2 days, – = negative after 14 days, + + = acid and gas after 1 day

The motility test should preferably be carried out at a temperature lower than 37 C, e.g. 30° C or room temperature.

test antisera O1–O157 diluted 1 100 showed that E1020/72 had a high titered reciprocal relationship to O25 Cross absorptions of antisera O25 and E1020/72 showed no decrease in homologous titres. Several other O test antigens reacted in E1020/70 antiserum, the highest titres, 320–640, being obtained with O115 and O129 No decrease in the titre (≳ 5120) of E1020/70 was seen after absorption with these antigens. E1020/70 was established as the test antigen of O158.

E2476/72 was negative in established O antisera and no test antigen reacted in the O antiserum prepared against E2476/72. The strain was established as test antigen of O159

E110/72 was negative in available O antisera, while test antigens O12 and O18 agglutinated to a titre of 320 in O antiserum of E110/72 After absorption of this serum with either of the two strains, no change was found in the homologous titre (≳ 5120) E110/72 was established as test strain of antigen O160

E223/69 agglutinated in antiserum O61 (titre 1280) while test antigen O61 was negative in the O antiserum of E223/69 No other O test antigens reacted to a titre above

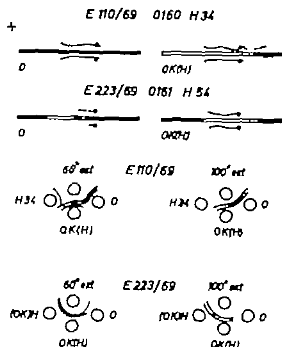


Fig 1 Comparisons of immunoelectropherograms and gel precipitation tests with crude extracts of E110/69 and E223/69 The troughs contain the homologous O and OK(H) antisera prepared with a 100° C and a non-heated formalinized *culture* respecti ely In the gel precipitation test the paper discs are soaked with the same antisera. The H54 antiserum prepared against a motile culture of E223/69 is denoted (OK)H. The H antiserum against which the E110/69 extracts are tested is H34 This antiserum is an (OK)H antiserum prepared against a motile culture of the test strain of antigen H34 (BP 12665) The wells above the trough and the upper paper discs to the left contain 60 C extracts which means supernatant from a culture heated to 60 C 20 min. The wells below the trough and the upper paper discs to the right contain the 60 C extracts heated to 100° C for 1 hour

160 in this antiserum and no absorptions were carried out. The 0 antigen of E223/69 was designated O161

The fifth strain, E480/68 was received as a new H antigenic strain belonging to O group 148 The O148 antigen of the strain was confirmed However the strain was unable to entirely deplete the O148 antiserum of O antibodies. In contrast, test antigen O148 removed all O antibodies from the E480/68 antiserum (OK antiserum as no O antiserum was prepared) According to this, the O antigen of E480/68 might be called O148⁻ (or O148a) because it did not posess the complete O148 antigen, as present in the test strain of O148 (or O148ab) In such situations strains may be designated by the same O antigen even though the antigens are not identical.

The H antigens of the strains were as follows E1020/72 belonged to H23 E2476/72 to H20 and E110/69 to H34 The H antigens of E223/69 and E480/68 were established as new types, H54 and H53 respectively None of the test antigens H1 to H53 reacted in H antiserum of E223/69 diluted 1 100 and a motile culture of E223/69 did not agglutinate in any of the established H antisera. Examination of the H antigen of E480/68 also gave negative results.

Examinations in O and OK(H) antisera by means of immunoelectrophoresis showed that four (E1020/72, E2476/72, E110/69 and E223/69) of the five strains gave anodic lines in both kinds of antisera and with both the 60 C extract and the 60 C extract further heated to 100 C (called 100° C extract) Ordinarily such lines are caused by the presence of acidic O antigens. No anodic K antigen lines could be separated from the O antigen lines. The absence of K lines was confirmed by examination in the double diffusion in gel. The reactions of two of the four strains are depicted in Fig 1 The precipitation lines obtained with O antisera are often much weaker than those in OK(H) antisera. This fact is clearly shown in the double diffusion test with E223/69 where the 100 C extract gives almost no reaction. In immunoelectrophoresis the same extract gives a line when tested against the O antiserum, but the line is weaker than that in the OK(H) antiserum.

An H precipitation line is rarely demonstrable in antisera using our methods In Fig 1 however an H line is seen in one of the examples. Strain E110/69 has H antigen H34 and in double diffusion in gel a line is demonstrable between the 60 C extract and antiserum H34 This line shows identity with

TABLE 2. *Results of Tube Agglutination Tests in O and OK(H) Antisera against E110/69, E223/69 and E480/68*

Antigen		O antiserum	OK(H) antiserum Unabsorbed	OK(H) antiserum Abs. by homologous culture heated to 100 C
E110/69	live[a)]	5120	5120	0
H34	100°C[b)]	5120	5120	0
	motile[c)]	0	640	640
E223/69	live	80	2560	0
	100°C	5120	5120	0
	motile	n.d.	5120	640
E480/68	live	n.d.	5120	5120
	Pril[d)]	n.d.	5120	0
	100°C	n.d.	1280	0

0 means titre < 20, 5120 means titre ≥ 5120
n.d. = not done.
a) Suspension from broth agar plate, formalinized.
b) Broth culture heated to 100° C for 1 hour
c) Motile broth culture of test strain of antigen H34 (orig. number BP12665)
d) Suspension from broth agar plate supplemented with 0.1 per cent fluid pril, formalinized.

a line between the same extract and the OK(H) antiserum of E110/72. No line is demonstrable with the 100 C extract. In immunoelectrophoresis a cathodic line is formed with the 60 C extract and the OK(H) antiserum, probably representing the H antigen. No H line is seen with the other strain, E223/69 depicted in Fig 1 either in the H (homologous (OK)H in this case) or in the OK(H) antiserum.

From the agglutination results shown in Table 2 it is seen that the OK(H) antisera of both E110/72 and E223/69 contain H agglutinins, a fact which justifies the designation OK(H) instead of OK antiserum. A motile culture of test antigen H34 agglutinates in OK(H) antiserum E110/69 absorbed by the homologous culture heated to 100 C, while the formalinized E110/69 culture (i.e. a formalinized suspension of a plate culture) fails to agglutinate. The same applies to the OK(H) antiserum of E223/69 the test culture of the newly established H53. Here the motile culture of E223/69 agglutinates in this antiserum absorbed by homologous boiled cultures, while a formalinized suspension of a plate culture of the same strain does not.

The fifth strain, E480/68 (O148:H54) had a cathodic O line and no anodic polysaccharide K antigen line. Similarly no K line was demonstrable in double diffusion in gel. In the OK(H) antiserum absorbed by homologous boiled culture the suspension of the plate culture agglutinated while the suspension prepared from a broth agar plate containing Pril® failed to do so. This fact indicated that the agglutination was due to flagella (1 4). The three OK(H) antisera (shown in Table 2) prepared by injecting rabbits with suspension from plate culture all contained H antibodies, but only one of the three plate cultures had flagella sufficiently developed to give an H agglutination.

The serotype formulae of the strains examined are summarized as follows. The antigens for which the strains will be test strains are italicized.

E10?0/72 = *O158* H23
E2476/72 = *O159* H20
E110/69 = *O160* H34

E223/69 = *O161 H54*
E480/68 = *O148.H53*

REFERENCES

1 *Döll W.:* Hemmung des Schwärmens von Proteus-Bakterien durch oberflächenaktive Substanzen (Pril und Rei) Zbl. Bakt. I Abt. Orig. *166* 43–47 1956.

2. *Kauffman F* The bacteriology of *Enterobacteriaceae* Munksgaard, Copenhagen 1966.

3 *Orskov F., Ørskov I Jann B. & Jann K* Immunoelectrophoretic patterns of extracts from all *Escherichia coli* O and K antigen test strains. Correlation with pathogenicity Acta path. microbiol. scand. Sect. B *79* 142–152 1971

4 *Ørskov I Ørskov F., Wittig W & Sweeney E. J.,* A new *E. coli* serotype O149:K91(B), K88ac(L):H10 *isolated from diseased swine.* Acta path. microbiol. scand. *75* 491–498, 1969

5 *Orskov I & Orskov F* The K antigens of *Escherichia coli* Re-examination and re-evaluation of the nature of L antigen. Acta path. microbiol. scand. Sect. B, *78* 593–604 1970

6 *Orskov I., Orskov F & Rowe B* Three new *Escherichia coli* O antigens O154 O155 and O156, and one new K antigen, K94. Acta path. microbiol. scand. Sect. B, *81* 59–64, 1973

7 *Rowe B. Escherichia coli* O148 and diarrhoea in adults. Brit. med. J Correspondence 3 741 1974

8. *Scheidegger J J* Une micro-méthode de l'immunoélectrophorèse Int. Arch. Allergy 103–110, 1955

cta path. microbiol. scand. Sect. B, 83 121–124 1975

TWO NEW *ESCHERICHIA COLI* O ANTIGENS, O162 AND O163, AND ONE NEW H ANTIGEN, H56 WITHDRAWAL OF H ANTIGEN H50

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Ørskov I., Ørskov F., Bettelheim, K. A. & Chandler M. E. Two new *Escherichia coli* O antigens, O162 and O163 and one new H antigen, H56. Withdrawal of H antigen H50. Acta path. microbiol scand. Sect. B, 83 121–124 1975

Three *E. coli* strains representing strains from faeces of normal persons were established as antigenic test strains of two new O antigens, O162 and O163 and one new H antigen, H56.

Key words: New *Escherichia coli* antigens O antigens H antigen.

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Epidemiological studies on normal intesti-l *Escherichia coli* flora have shown that me strains cannot be typed as to O and H ntigens. Antisera prepared against some such rains have demonstrated the frequent pre-nce of these in the environment and thus stified the establishment of new test anti-ns.

MATERIAL AND METHODS

Three *E. coli* strains were received at the WHO Collaborative Centre for Reference and Research on Escherichia from *K. A. Bettelheim*. Strain 10B1/1 was representative of strains isolated from faecal pecimens of mothers and babies delivered in a maternity unit (1). Strains SN3B/1 and SN2N/1 represented strains which had been isolated repeatedly from healthy adults.

Serological examination. The methods were those used previously: agglutination technique and antiserum production as described by *Kauffmann* (4) immunoelectrophoresis as described by *Scheidegger* (9) and extract procedure according to *Ørskov et al.* (6)

RESULTS

The fermentation reactions are given in Table 1

Examination of strain 10B1/1 in all O test antisera 1 100 was negative. An O antiserum raised against 10B1/1 agglutinated in one laboratory test antigens O76 and O101 to the titres 640 and ≧ 5120 respectively. The titre of strain 10B1/1 was ≧ 5120 and remained the same after absorption with the O76 and O101 strains. Strain 10B1/1 was also found to be related to O101 in the other laboratory but the cross reaction was weak. 10B1/1 was established as test antigen of O antigen O162.

Strain SN3B/1 cross-reacted with O75 The titres for the cross reactions were 640 in one laboratory Before, as well as after absorption with the cross-reacting strain the

TABLE 1 *Biochemical Properties of Strains Examined*

	10B1/1	SN3B/1	SN2N/1
Adonitol	+	—	—
Dulcitol	—	+	—
Sorbitol	+	+	+
Raffinose	—	+	—
Xylose	+	+	+
Rhamnose	+[3]	+	+
Maltose	+	+	+
Salicin	—	—	—
Inositol	—	—	—
Lactose	+	+	+
Sucrose	—	+	—
Sorbose	—	—	—
Mannitol Gas	+ +	+ +	+ +
Glucose, Gas	+ +	+ +	+ +
Indol	+	+	+
H_2S	—	—	—
Gelatin	—	—	—
Ammonium glucose	+	+	+
Ammonium citrate	—	—	—
KNO_3	+	+	+
Voges-Proskauer	—	—	—
Methyl red	+	+	+
Urease	—	—	—
KCN	—	—	—
Malonate	—	—	—
Motility 37 C	+	+	(+)*
Haemolysis	—	—	—
Serotype	O162 H10	O163 H19	O139 H56

SN2N/1 is hardly motile at 37 C. The motility test should preferably be carried out at a lower temperature, e g 30 C or room temperature.

TABLE 2. *O Antigenic Relationship Between Test Strain of O Antigen O75 and Strain SN3B/1 Demonstrated by Tube Agglutinatio Test*

Antigen	O antisera against			
	O 5		SN3B/1	
	Unabs.	Abs. by SN3B/1 100 C	Unabs.	Abs. by O75 100 C
O75	≧5120	≧5120	1280	<20
SN3B/1	640	<20	≧5120	≧5120

homologous titres of both antisera were ≧ 5120 In the other laboratory the relationship between the two strains was only slight. From Table 2 it will be seen that both strains might be designated as O75 strains, with a common and a special O antigen factor each. However the O antigen of strain SN3B/1 was designated O163 because the reaction in O75 antiserum was weak in one of the laboratories.

The H antigens of the two strains were as follows 10B1/1 had H10 and SN3B/1 had H19

The O antigen of the third strain, SN2N/1, was designated O139 In both laboratories the strain agglutinated in O139 antiserum with a titre close to that of the test strain O139 It might be added however that the O antigen of SN2N/1 was not identical with the O antigen of test strain O139 as demonstrated by cross absorptions of antisera against the two strains.

The H antigen of SN2N/1 was established as a new H antigen, H56. A motile culture of the strain did not react to any significant degree in any known H antiserum diluted 1 100 and only minor reactions were found with H24 in one laboratory and with H11 and H42 in the other laboratory when the H test antigens were examined in an H antiserum against SN2N/1 It should be added that the motility test in the case of strain SN2N/1 should be carried out at a temperature below 37 C, e g 30° C or room temperature, as it is very difficult to demonstrate motility at 37 C in semisolid medium in this strain.

All three strains were considered to be devoid of K antigens. They were all as agglutinable in the O antiserum as in the OK antiserum (produced with non-heated culture) After absorption of the OK sera with homologous 100 C culture these sera were tested with suspensions both from a normal broth agar plate and from one to which Pril® had been added. In two cases culture from the non-supplemented plate agglutinated in the absorbed OK antiserum, while the suspension from the Pril plate did not indicating that the agglutination was due to flagella (2, 8)

In examination by immunoelectrophoresis, strain 10B1/1 showed O antigen precipitation lines on the cathodic side of the basin, while

SN3B/1 and SN2N/1 had O lines moving towards the anode. No separate K antigen lines could be distinguished in any of the three strains.

The serotype formulae of the three strains are given below The antigens for which the strains will be test strains are italicized

10B1/1 *O162* H10
SN3B/1 *O163*.H19
SN2N/1 O139.*H56*

In "Identification of Enterobacteriaceae" by *Edwards & Ewing* (3) the last two H antigens of *E coli* are "H50" (6226-60) and "H51" (669-58) In 1971 *Lautrop et al.* (5) published a paper on H_2S producing *E. coli* strains Two new H types were represented among these strains. They were established as H51 (C218-70) and H52 (C2187 70). The test strain of the new O antigen, O151 had a new H antigen which was numbered H50 (7) By mistake the American "H50" and H51 strains (6226-60 and 669-58) were not sent for confirmation to the WHO Centre on Escherichia until after their publication, and an unacceptable situation existed with different H antigens represented by the same numbers. By examination of the American "H50" strain it was shown that it had an H antigen so strongly related to H52 (2187 70) that it should be disregarded as a new H type strain. The H antigen of the American "H51" strain (669-58) was confirmed as a new type but shall have to be renamed H55 instead of H51 since that number has been given to strain C218-70 The O antigen of strain 669-58 was claimed to be O126 but has become rough. Because of the disadvantage of having a rough (O antigen missing) H antigen type strain it will be replaced by a smooth strain with H55 but probably with another O antigen than O126 (*B Rowe* personal communication) This means that both "H50" (6226-60) and "H51" (669-58) in agreement with *Dr Ewing* will be cancelled as H test strain in the next edition of "Identification of Enterobacteriaceae

In the present paper we shall further have to withdraw the H antigen H50 (880-67) (7) There are two reasons for doing so. In the first place H50 has a strong relationship to H10 (Bi623-42) which was overlooked probably because we find that the reaction of H50 with antiserum H10 varies to a greater extent than usually seen. In some cases, though not regularly H50 cultures are able to absorb most H antibodies from the H10 antiserum. Secondly it is becoming more and more difficult to obtain sufficiently motile cultures of H50 Strain 880-67 is maintained as test strain of O antigen O151 The serotype of this strain will now be O151 H10

The fate of the published H50 and H51 strains is summarized as follows

880-67 O151 H50
Ørskov et al. (1972) Changed to O151 H10 (still type strain of O151)

6226-60 O86a.H50
Edwards & Ewing (1972) Cancelled

C218-70 O8, O60.H51
Lautrop et al. (1971) Maintained

669-58, O126.H51
Edwards & Ewing (1972) Changed to rough H55 will be replaced by another strain

Only 880-67 has been examined for K antigen which could not be demonstrated.

DISCUSSION

The present investigation illustrates the fact that the variation in agglutination results from one laboratory to the other may result in the establishment of new test antigens. Strain SN3B/1 would be designated as a strain strongly related to O75 according to the routine examination in one of the laboratories, not in the other Non-identity revealed by cross absorption tests between test strain for O75 and SN3B/1 justified the establishment of the new O antigen, O162. Strain SN2N/1 reacted in antiserum O139 in both laboratories and was therefore serotyped as O139 even though it was not identical with test antigen O139 according to cross absorption test. However it is a well-known fact that when a serotype diagnosis is given to a

strain this does not mean that the antigen of the strain and the corresponding test antigen are identical.

REFERENCES

1 *Bettelheim K. A Tech-Chan Ching, H Chandler M E., O'Farrell, S M Rahamin L., Shaw E. J & Shooter R A.*. Further studies of *Escherichia coli* in babies after normal delivery J Hyg (Lond.) *73* 277–285 1973

2. *Dõll W* Hemmung des Schwärmens on Proteus-Bakterien durch oberflächenaktive Substanzen (Pril und Rei) Zbl. Bakt. I. Abt. Orig. *166* 43–47 1956.

3 *Edwards P R. & Ewing, W H* Identification of *Enterobacteriaceae* 3 ed. Burgess Publishing Company 1972, p. 92.

4 *Kauffmann F.*. The bacteriology of *Enterobacteriaceae* Munksgaard, Copenhagen 1966.

5. *Lautrop H., Ørskov I & Gaarslev K.*. Hydrogensulphide producing variants of *Escherichia coli*. Acta path. microbiol. scand. Sect. B, *79* 641–650 1971

6 *Ørskov F Ørskov I., Jann B. & Jann, K* Immunoelectrophoretic patterns of extracts from all *Escherichia coli* O and K antigen test strains. Correlation with pathogenicity Acta path. microbiol. scand. Sect. B, *79* 142-153, 1971

7 *Ø sk F., Ørskov I & F rennes, A J* Four new *Escherichia coli* O antigens, O148, O151, O152, O153 and one new H antigen, H50, found in strains isolated from enteric disease in man. Acta path. microbiol. scand Sect. B, *80* 435–440, 1972

8. *Ørskov I., Ørskov F., Wittig, W & Sweeney, E. J.*. A new *E. coli* serotype, O149 K91(B) K88ac(L) H10, isolated from diseased swine. Acta path. microbiol. scand. *75* 491–498, 1969

9 *Scheidegger J J* Une micro-méthode de l'immunoélectrophorèse. Int. Arch. Allergy 7 103–110 1955

cta path. microbiol. scand. Sect. B, 83 125–128, 1975

RADIOIMMUNOASSAY OF HEPATITIS B ANTIGEN AND ANTIBODY IN DIALYSIS AND TRANSPLANT PATIENTS

A Five-Year Follow Up Study

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i7 dialysis patients, 29 of whom had been exposed to hepatitis in 1968–69 were re-examined or hepatitis B antigen (HB-Ag) and antibody (HB-Ab) by means of radioimmunoassay (RIA) nd counter immunoelectrophoresis (CIE) Infected patients were followed for five years. Compared to CIE, RIA did not detect additional HB-Ag-positive patients, but antibody could persistently be detected in seven out of fifteen patients previously supposed to have escaped infection. Nine surviving HB-Ag-positive transplant patients remained carriers for 50–72 months. None developed clinical signs of chronic hepatic failure.

Key words Hepatitis B antigen; dialysis and transplant patients follow up study

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The discovery of HB-Ag added greatly to our understanding of the epidemiology of viral hepatitis in dialysis units (4 5 10) However in most studies, the laboratory techniques used have not been sufficiently sensitive to detect infection in a high proportion of infected individuals. This applies particularly to the detection of hepatitis B antibody (HB-Ab) which could only be detected sporadically after recovery from acute hepatitis B.

The development of a radioimmunoassay (RIA technique for detection of HB-Ag and HB-Ab brought about that we found it appropriate to re-investigate stored serum samples from a series of patients exposed to a hepatitis outbreak in a dialysis unit in 1968–69 in order to evaluate whether results previously obtained by the less sensitive gel diffusion and electrophoretic methods were valid (9) and to perform a five year follow up of the patients infected with hepatitis B virus.

MATERIAL AND METHODS

Summary of the Outbreak and Previous Results

The clinical outbreak of viral hepatitis occurred from August 1968 to June 1969 during which period two dialysis patients and one transplant patient died in hepatic coma. Examination in November 1969 of stored serum samples showed that

TABLE 1 *Composition of the Series 1969*

	No of patients	HBAg and HBAb negative	HBAg positive		HBAb positive	
			CIE	RIA	CIE	RIA
Haemodialysis patients						
Exposed	8	3 (2)*	0	0	1	5
Non exposed	9	9	0	0	0	0
Transplant patients						
RH patients§						
Exposed	21	6	12	12 (3)	0	3 (1)
Non exposed	11	11	0	0	0	0
Referred patients	8	7	0	0	0	1

* Figures in parantheses indicate the exposed patients not alive at the follow up in 1974

§ RH Rigshospitalet.

the dialysis unit had been contaminated with HB-Ag from April 1968 (earliest sample available) to August 24th 1969 when the last of the HB-Ag positive patients underwent transplantation (9)

Patients and Material

Retesting study The series comprised 57 patients treated during the four months period from November 1969 to March 1970 At least two stored serum specimens from each patient were re-examined by means of counter immunoelectrophoresis (CIE) and radioimmunoassay (RIA)

The series was divided into groups of "exposed" and "non exposed" patients.

The exposed group comprised 29 patients who had been on haemodialysis before elimination of HB-Ag

The "non exposed group comprised 28 patients who started treatment after elimination of HB-Ag from the dialysis unit. Nine patients were on the dialysis program and eight were referred from other dialysis units for transplantation.

Follow up investigation. In April 1974 23 exposed survivors were examined for HB-Ag and HB-Ab by means of CIE and RIA (see Table 1) Six out of eight dialysis patients were alive. Five had a functioning kidney transplant one had been re-admitted to dialysis treatment one year after transplantation due to graft failure. Seventeen of the 21 transplant patients were alive with a functioning graft

Both in dialysis patients and in transplant recipients, liver function had been routinely controlled by measurement of serum glutamic-pyruvic transaminase (S-GPT) levels and examination for HB-Ag by means of CIE.

Detection of HB-Ag and HB-Ab

Investigation for HB-Ag and HB-Ab was performed by counter immunoelectrophoresis (8) and by a radioimmunoassay (7) Compared with CIE, the sensitivity of the latter was approximately 50 times higher for antigen detection and 4000 times higher for demonstration of antibody The specificity of the HB-Ab results obtained by RIA was confirmed by absorption test using purified HB-Ag and HB-Ag containing sera

RESULTS

Retesting Study

The results of the re-examination of stored serum samples are summarized in Table 1

Retesting by means of CIE gave results identical to those previously obtained and RIA did not detect other HB-Ag positive patients. However the radioimmunoassay detected HB-Ab in seven exposed patients whose sera were negative by immunoelectrophoresis. An analysis did not reveal any correlation to the time of dialysis of the infected patients during the period of contamination. Serological evidence of infection was not observed in any of the patients who had not been exposed to hepatitis.

The non-exposed transplant recipient whose serum contained HB-Ab had one year earlier and in another dialysis centre, had an icteric hepatitis.

ollow Up Study

By April 1974 six of the 29 patients in the xposed group had died (Table 1)

The 12 patients who in 1969 were HB-Ag ositive remained positive, three of these until heir death (1970 1971 and 1972 respectively) In no case, the fatal course was associated with impairment of liver function.

In one patient, the CIE test changed to egative two years after transplantation, but ntigen could be detected by RIA at the ollow up. Including results from stored erum samples, persistent HB-Ag-carriage hroughout 56–72 months was thus demonstrated in all of the surviving patients. During he follow up period, 10 out of the 12 HB-Ag positive patients showed borderline levels, r slightly elevated S-GPT levels, for several ears, indicative of chronic liver affection. Liver biopsy was performed in two patients oth specimens showed chronic aggressive epatitis. One patient developed a clinical cteric hepatitis during cytotoxic treatment for sarcoma five years after transplantation. None of the remaining patients developed linical signs of hepatic failure.

The eight surviving HB-Ab-positive paients showed all persistent, positive reaction for HB-Ab. Four of these patients had had eriods of slightly elevated serum transaminase levels during the follow up period.

Slightly elevated S-GPT values of long duration were also observed in three of nine HB-Ab-negative patients.

None of the originally negative patients developed antigen or antibody Co-existence f HB-Ag and HB-Ab was not observed.

DISCUSSION

Most investigations of HB-Ag in haemodiasis associated hepatitis have been performed n laboratory techniques of low (gel diffuson) or medium (CIE) sensitivity

In the present study unknown HB-Ag arriers were not detected by the 50-fold nore sensitive RIA, confirming our previous onclusion that HB-Ag had been eliminated rom the dialysis unit.

Persistence of HB-Ag for up to six years, was demonstrated in all of 12 infected transplant patients.

None of the HB-Ag positive transplant patients developed clinical signs of chronic hepatic failure during the follow up period although most of them had biochemical signs of chronic hepatitis. This finding suggests a favourable course of the disease in the immunodepressed patient similar to that described for other patients with chronic hepatitis and persistent antigenaemia (2)

A perplexing problem has been the inability to detect antibody in serum from patients who have recovered from viral hepatitis. However the development of the more sensitive techniques has recently permitted the detection of HB-Ab in a high proportion of such patients and also at varying frequency in population studies (3)

In uraemic patients, the humoral antibody response is decreased (11) thereby offering an explanation of the tendency to chronic antigenaemia. However In spite of the impaired immunological responsiveness, serum from one of the dialysis patients contained HB-Ab in very high titres, and by RIA, HB-Ab could be detected in one half of the exposed HB-Ag negative patients. Similar results were obtained by *Desmyter et al.* (1) and *Szmuness et al* (6) while development of antibody in immunosuppressed transplant patients has not yet been reported.

The detection of HB-Ab in seven patients who previously had been considered to have escaped infection during the outbreak demonstrated that immunization had contributed significantly to the elimination of HB-Ag from the dialysis unit and thus modified our previous conclusion concerning the effeciency of the prophylactive measures (9)

The finding of HB-Ab exclusively in previously exposed patients and the demonstration of its persistence for five years even in these immunosuppressed patients provides clinical evidence of the fact that demonstration of this antibody by RIA is a specific long term marker of previous exposure to hepatitis B.

Addendum

After submission of the present paper *Chatterjee S N., Payne J E. Buckel M D Redeker A G & Bernet V* (New Engl. J Med. *291* 62–65 1974) in a similar study have confirmed our previous observation (9) that transplantion of HB-Ag carriers does not seem to imply any increased operative risk or a deterioration of the disease.

REFERENCES

1 *Desmyter J Fiasse R., Verberckmoes R., Vandenbroucke J M., Strikon C v Y de Dive C & Michielsen P* Anti-HAA immunity in renal units with a high and low incidence of serum hepatitis. In: Cameron, J S. (Ed.) Proc. Europ. Dial. Transpl. Ass. *9* 84–89 1972.

2. *Dietrichson O Nielsen J O., Elling, P & Christoffersen P.* The relevance of a serological classification of chronic hepatitis. Acta path. microbiol. scand. Sect. B, *81* 519–524 1973

3 *Lander J J., Holland, P V Alter H J Chanock R M & Purcell R. H* Antibody to hepatitis-associated antigen. Frequency and pattern of response as detected by radioimmunoprecipitation. J Amer med. Ass. *220* 1080–1082 1972.

4 *London W T., DiFiglia, M., Sutnick A T & Blumberg, B. S* An epidemic of hepatitis in a chronic-hemodialysis unit. Australia antigen and differences in host response. New Engl. J Med. *281* 571–578, 1969.

5 *Nordenfelt E. Lindholm, T & Dahlquist E.* A hepatitis epidemic in a dialysis unit. Occurrence and persistence of Australia-antigen among patients and staff. Acta path. microbiol. scand. Sect. B, *78* 692–700 1970.

6 *Szmuness W., Prince A. M., Grady G. F et al.* Hepatitis B infection. A point-prevalence study in 15 US hemodialysis centers. J Amer med. Ass. *227* 901–906, 1974

7 *Skinhøj P & Hansen J F.* Preparation and characterization of hepatitis B antigen for radioimmunoassay Scand. J clin. Lab. Invest. *32*: 199–203, 1973

8. *Skinhøj P & Thalstrup H.* Hepatitis-associated antigen. Qualitative and quantitative determination. Scand. J clin. Lab. Invest. *27* 61–66 1971

9 *Steiness I & Skinhøj P.* Hepatitis associated antigen Elimination from a dialysis unit and persistence in renal transplant recipients. Acta path. microbiol. scand. Sect. B, *79* 721–724 1971

10. *Turner G C & White G B. B.* S.H. antigen in haemodialysis-associated hepatitis. Lancet *ii* 121–125 1969

11 *Wilson W E. Kirkpatrick C G. & Talmage D W.* Suppression of immunologic responsiveness in uraemia. Ann. intern. Med. *62* 1–13 1965.

cta path. microbiol. scand. Sect. B *83* 129–132, 1975

A FIMBRIATED STRAIN OF *PASTEURELLA MULTOCIDA* WITH SPREADING AND CORRODING COLONIES

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Henriksen, S. D. & Frøholm, L. O. A fimbriated strain of *Pasteurella multocida* with spreading and corroding colonies. Acta path. microbiol. scand. Sect. B, 83 129–132, 1975

A strain identified as *Pasteurella multocida* (dog type") grew on blood agar with spreading and corroding colonies. It was found to be fimbri ted. A non-fimbriated variant of the strain which did not produce spreading and corroding colonies was isolated. The fimbriated strain regularly formed surface growth by static incubation in broth. The mode of surface translocation of this strain appeared to be twitching

Key words *Pasteurella multocida* fimbriated strain spreading and corroding colonies.

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The purpose of this paper is to report the olation of an unusual strain of *P multocida* om a human middle ear

Case history The patient was a 47 years old oman who had suffered from chronic otitis media n the right side continuous secretion had persisted for 35 years, associated with gradual eczematous changes of the skin. She now visited an ar-nose-throat specialist, complaining of an acut xacerbation of the condition and increased secreion. Pus from the right ear yielded abundant rowth of the strain to be described nd a few olonies of white staphylococci.

METHODS

Conventional methods were used for the study of morphological, cultural and biochemical characters of the strain.

Electron microscopy was carried out as described previously (3) Agar plate microscopy was carried out as described by *Henrichsen* (6)

Transformation The methods described by *Berry* (1) including continuous DNA-exposure, as well as number of modifications were tried, including the use of different media with and without serum and with and without ddition of calcium ions, recipient cultures of different age, different times of DNA-exposure and phenotypic expression, and different concentrations of streptomycin for transformant selection. In view of the negative results, details do not seem necessary

RESULTS

Microscopy The strain consisted of tiny gram-negative coccoid and coccobacillary cells with a few short rods and occasional thin filaments.

Colonies After about 20 h, colonies were about 1 mm in diameter they were round, low convex, smooth, shiny but not mucoid. There was no change of the medium. After 2 days in a humid atmosphere at 35 C, the size increased to about 2 mm in diameter The colonies appeared to have sunk slightly



Fig 1 Spreading and corroding colonies on blood agar incubated in a humid atmosphere for 7 days, × 4





down in the agar a narrow shallow groove being in evidence along the margin. Rather deep irregular depressions became apparent when the colonies were scraped off After about 3 days, thin zones of spreading growth started to appear around the colonies and in the following days, the zones increased to considerable size (Fig 1) Corrosion of the agar under the zones was not noted.

From a blood agar culture of the strain, a non-spreading variant was isolated after subcultivation of growth bulging from the margin of a colony on agar base without blood. The colonies of the variant resembled those of the original strain, but did not appear to sink down in the agar when they were scraped off only very shallow smooth, just visible depressions, but no "corrosion" were seen and, on prolonged incubation, no

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Fig 2 Cells of spreading-corroding variant showing fimbriae (upper) and of non-spreading variant, without fimbriae (lower) Optical differentiation used (2) × 60,000

ig zones appeared. Otherwise the two [variants] appeared to be identical.

Growth characteristics. The strains ap[peared] to be somewhat fastidious and failed [to] grow on some of the simpler fluid media, [e.]g. Clark & Lub's medium and nitrate broth. Growth on nutrient agar was fair. No growth occurred at room temperature. The strains were facultative anaerobes. In stationary cul[tures] in fluid media, a marked tendency of [the] spreading strain to form surface pellicles was noted.

Biochemical reactions. The following tests were positive: Catalase, oxidase, acid production (fermentative) from glucose, galactose, xylose (weak) and sucrose. Indole production.

The following tests were negative: Fermentation of lactose, maltose, rhamnose, arabinose, mannitol, sorbitol, and dulcitol, production of H_2S (TSI-medium), urease, phenylalanine deaminase, liquefaction of gelatine and coagulated serum.

These reactions appear to be compatible with a classification as *P. multocida*, the "dog type" as described by *Frederiksen* (5). Both strains were sensitive to penicillin and to other commonly used antibiotics.

Electron microscopy. About one in ten cells of the corroding and spreading variant was found to be fimbriated, but the cells of the non-corroding, non-spreading variant could not be shown to have fimbriae when several hundred were carefully examined (Fig. 2).

Agar microscopy. On media without blood spreading zones were only irregularly seen; the small size of the cells and the very narrow dimensions of the spreading zones observed made it difficult to see twitching motility. In some zones, however, particularly around miniature colonies, an arrangement of the cells typical of twitching was seen (Fig. 3). Also the appearance of the spreading zones was strikingly similar to that of zones produced by *Moraxella* strains, an appearance which is due to twitching.

Transformation. All attempts failed to transform any of the two variants, using DNA from streptomycin resistant mutants of both variants.



Fig. 3. Miniature colonies of non-spreading (right) and of spreading variant (left). The arrangement of the cells around the latter colony suggests twitching. × 1,000.

DISCUSSION

We have not found any previous description of strains of *P. multocida* producing spreading and corroding colonies. As in the *Moraxella* species *M. nonliquefaciens*, *M. bovis* and *M. kingae* (2, 4, 7) this trait was associated with fimbriation and, also as in the case of *Moraxella*, it was possible to isolate a non-spreading, non-corroding and unfimbriated variant.

The spreading zones produced by the *Pasteurella* strain were very like those caused by twitching of the mentioned *Moraxella* species and, although we did not succeed in actually seeing twitching cells, we feel that the arrangement of the cells in some spreading zones indicates that they were actually produced by twitching.

Contrary to expectation, competence in transformation was not demonstrated in the spreading/corroding strain. This might indicate that fimbriation in this strain is not associated with competence, as in *Moraxella* species, but it cannot be precluded that the fimbriae may not have been of the right type or simply that we failed to find conditions suitable for transformation.

It may be mentioned that previous experiments (8) have failed to detect competence

in strains of *P multocida*, although strains of other *Pasteurella* species appeared to be transformable. Corroding and spreading was not detected in any of those strains.

The isolation of the strain described in this paper shows that the association of spreading/corroding colonies with twitching and fimbriation is not a property of *Moraxella* species exclusively but may also be found in *P multocida*.

It may be emphasized that such phenomena may remain unobserved unless cultures are incubated for periods of time longer than one or two days. Incubation in a humid atmosphere may also be important, although systematic studies of this point have not been carried out.

REFERENCES

1 *Bøvre K* Studies on transformation in *Moraxella* and organisms assumed to be related to *Moraxella*. 1 A method for quantitative transformation in *Moraxella* and *Neisseria* with streptomycin resistance as the genetic marker Acta path. microbiol scand. *61* 457–473 1964

2. *Bøvre K., Bergan T & Frøholm L. O.* Electron microscopical and serological characteristics associated with colony type in *Moraxella nonliquefaciens*. Acta path. microbiol scand. Sect. B, *78* 765–779 1970.

3 *Bøvre K & Frøholm L. O* Variation in colony morphology reflecting fimbriation in *Moraxella bovis* and two reference strains of *M nonliquefaciens*. Acta path. microbiol scand. Sect. B, *80* 629–640, 1972.

4 *Bøvre K & Frøholm, L. O* Competence in genetic transformation related to colony type and fimbriation in three species of *Moraxella*. Acta path. microbiol. scand. Sect. B, *80* 649–659 1972.

5 *Frederiksen W* *Pasteurella* taxonomy and nomenclature. In Winblad, S. (Ed): Contributions to microbiology and immunology. 2 *Yersinia, Pasteurella* and *Francisella*. Karger, Basel 1973 pp. 170–176.

6. *Henrichsen J* Bacterial surface translocation. A survey and a classification. Bacteriol. Rev *36* 478–503 1972.

7 *Henrichsen, J Frøholm L. O & Bøvre, K.* Studies on bacterial surface translocation. II Twitching motility associated with fimbriation in colony variants of *Moraxella nonliquefaciens, M bovis* and *M kingii*. Acta path. microbiol. scand. Sect. B, *80* 445–452, 1972.

8. *Henriksen S D* Transformation of streptomycin resistance in Pasteurella. Acta path. microbiol. scand. *55* 496, 1962.

ta path. microbiol. scand. Sect. B 83: 133–140 1975

TWITCHING MOTILITY AND POSSESSION OF POLAR FIMBRIAE IN SPREADING *STREPTOCOCCUS SANGUIS* ISOLATES FROM THE HUMAN THROAT

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Henriksen, S. Dick & Henrichsen, J. Twitching motility and possession of polar fimbriae in spreading *Streptococcus sanguis* isolates from the human throat. Acta path. microbiol. scand. Sect. B, 83 133–140, 1975

A collection of 19 strains of alpha haemolytic streptococci, isolated from throat swabs and characterized by production of spreading zones around colonies on blood agar, was found to constitute a very homogeneous group with morphological, physiological and biochemical characters corresponding to those of streptococci of sero-group H, or *Streptococcus sanguis* and they all appeared to possess the group H antigen. They all had a common agglutinogen and, in addition, heterogeneous agglutinogens. The spreading growth, which appears to be a common property of *S. sanguis* was due to twitching motility and the spreading cultures possessed polar fimbriae. Neither twitching motility nor the possession of polar fimbriae have been observed in gram-positive bacteria before.

Key words: *Streptococcus sanguis* spreading growth; twitching motility polar fimbriae.

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This study started with an accidental obser tion a throat culture which had been left in e incubator in a humid atmosphere for sev al days, was found to contain a number of lonies which were surrounded by wide, thin reading zones, similar to the zones pro- uced by some species of *Moraxella* The or anisms were found to be alpha haemolytic reptococci. In view of the considerable in- rest connected with surface translocation of acteria (4) it was decided to search for dditional strains of this kind of streptococcus nd in the course of a short period of time, 9 such strains were collected. It is the purpose of this paper to report the results of studies undertaken in order to clarify the nature and taxonomic significance of this spreading phenomenon.

MATERIAL AND METHODS

The strains were isolated from cultures of throat swabs on 5 per cent human blood agar plates supplied with streaks of *Staphylococcus aureus* to facilitate growth of haemophilus. The cultures were incubated in closed plastic containers, containing water in the bottom, for 4 to 5 days or more at 35 C Alpha haemolytic streptococci with spreading colonies were isolated from 19 out of 122 cultures, an incidence of 15.5 per cent. Each

in strains of *P multocida* although strains of other *Pasteurella* species appeared to be transformable. Corroding and spreading was not detected in any of those strains.

The isolation of the strain described in this paper shows that the association of spreading/corroding colonies with twitching and fimbriation is not a property of *Moraxella* species exclusively but may also be found in *P multocida*.

It may be emphasized that such phenomena may remain unobserved unless cultures are incubated for periods of time longer than one or two days. Incubation in a humid atmosphere may also be important, although systematic studies of this point have not been carried out.

REFERENCES

1 *Bøvre K.* Studies on transformation in *Moraxella* and organisms assumed to be related to *Moraxella*. 1 A method for quantitative transformation in *Moraxella* and *Neisseria* with streptomycin resistance as the genetic marker Acta path. microbiol. scand. *61* 457–473 1964

2. *Bøvre K. Bergan T & Frøholm, L. O* Electron microscopical and serological characteristics associated with colony type in *Moraxella nonliquefaciens*. Acta path. microbiol. scand. Sect. B, *78* 765–779 1970.

3 *Bøvre K. & Frøholm L. O* Variation in colony morphology reflecting fimbriation in *Moraxella bovis* and two reference strains of *M nonliquefaciens*. Acta path. microbiol. scand. Sect. B, *80* 629–640, 1972.

4 *Bøvre K & Frøholm L. O.* Competence in genetic transformation related to colony type and fimbriation in three species of *Moraxella* Acta path. microbiol. scand. Sect. B, *80* 649–659 1972

5 *Frederiksen W.* *Pasteurella* taxonomy and nomenclature. In Winblad, S. (Ed): Contributions to microbiology and immunology. 2 *Yersinia Pasteurella* and *Francisella*. Karger, Basel 1973 pp. 170–176.

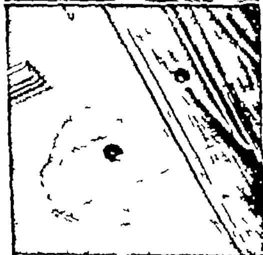
6. *Henrichsen J* Bacterial surface translocation. A survey and a classification Bacteriol. Rev *36* 478–503, 1972

7 *Henrichsen J., Frøholm, L. O & Bøvre, K* Studies on bacterial surface translocation II Twitching motility associated with fimbriation in colony variants of *Moraxella nonliquefaciens M bovis* and *M kingii*. Acta path. microbiol. scand. Sect. B, *80* 445–452, 1972

8. *Henriksen S D.* Transformation of streptomycin resistance in Pasteurella. Acta path microbiol. scand *55* 496, 1962.







of Parafilm®. A formvar coated carbon reinforced copper grid was placed on top of this drop, removed after 30–60 s, and excess liquid culture was sucked off. Bacteria remaining on the grid were stained by placing the grid on top of a drop of 1 per cent (w/v) ammonium molybdate (adjusted to pH 7.4 with NH_4OH) for another minute, removing it and sucking off excess stain.

Electron microscopy was carried out at 60 kV on a Philips EM 200 electron microscope at primary magnifications of 9,000 ×. Negatives were obtained on Kodak Fine Grain Release Positive Film Type 5302 and suitable fields were photographically enlarged as desired.

Measurements of fimbrial diameters were performed on isolated fimbriae as described elsewhere (5).

RESULTS

Morphology. The cells were small non-motile gram-positive cocci, mostly spherical, including some elongated cells and some pleomorphism. In films from blood agar the cells were mainly arranged singly and in pairs. In films from Todd-Hewitt broth, they were arranged in pairs and very short chains, seldom more than 6 to 8 cells per chain. Colonies on blood agar were small after 20–24 h, pinpoint size to c. 0.5 mm. After 2 or 3 days, the size increased to c. 1 mm. The colonies were surrounded by distinct zones of partial haemolysis with peripheral greening. After 3–5 days, narrow thin spreading zones started to appear around the colonies. The zones enlarged during the following days until they became fairly large and more or less irregular, often with dentate margins. Examples of dimensions of spreading zones are 20 × 8 mm, 15 × 7 mm and 13 × 6 mm. The zones were measured in 7 days old cultures. The long axis of the zone was always along the streak (Fig. 1).

The occurrence of spreading was irregular varying from one culture to the next. Some cultures did not show any zones, others showed zones of moderate or large size. The reasons for these variations were not studied system-

Fig. 1. Spreading colonies on blood agar after incubation for 7 days, × 4. Upper: Strain 419. Middle: Strain 445. Lower: Strain 736.



Fig 2 Strain 13843 Peripheral part of spreading zone on Lewinthal agar plate incubated at 35 C for 72 hours, approx. 300 ×

atically but the impression was that they might be due to variations in the media (length of storage before use?) or to growth conditions (degree of humidity of the atmosphere?) In any case, there was a marked tendency for all cultures inoculated on the same day to show similar degrees of spreading

The spreading zones were similar to those produced by strains of *Moraxella nonliquefaciens M kingae* or *M bovis* in the spreading-corroding phase (8 9 11) which are due to twitching (7) but "corrosion was not detected under the zones.

Repeatedly it was noted that contaminants in the cultures, e.g white staphylococci stimulated the growth of the streptococci, as shown by increased size, both of colonies and of spreading zones.

One of the two reference group H strain (NCTC 7863) repeatedly showed spreading zones, whereas the second (NCTC 7863) never did.

Determination of the Kind of Surface Translocation

Five strains were studied by agar plate microscopy viz. strains 13843 14241 1449, 14502 and 14506. Among these strain 14506 did not show spreading zones on either Lewinthal or ascites agar plates in one of our laboratories, and microscopically there was no sign of surface translocation. This strain had regularly produced large spreading zones in the other laboratory The reasons for the behaviour of this strain (change after freeze drying? dissociation? contamination?) remain to be studied.

The other 4 strains formed small spreading zones on ascites agar plates and rather pronounced spreading on Lewinthal agar plates. The micromorphological patterns and the manner of cell movement were typical of twitching motility i.e. the cells of the outermost part of the spreading zone lay loosely scattered over the agar surface (Fig 2) and single cells or pairs of cells could be seen to move by small leaps.

Electron Microscopy

The same 5 strains that were examined by agar plate microscopy were also examined in the electron microscope. No fimbriae were observed with certainty on strain 14506, but the other 4 strains all possessed *fimbriae*, although only on a minority of the cells in the preparations. Fig 3 shows two cells of strain 13843 Fimbriae with a diameter of approximately 40 Å can be seen originating from the pole of one of the cells as well as from the region of connection between the cells.

Physiological and Biochemical Characters

All strains grew well in Todd Hewitt broth, sometimes slowly at the start but finally pro-



Fig 3 Strain 13843 A pair of cells, both of which have started to divide Fimbriae with a diameter of approx. 40 Å originate from the pole shown and from the region of connection (arrows) Negatively stained with 1 per cent ammonium molybdate pH 7 4 90,000 ×

ducing heavy turbidity within 48 h, with a somewhat viscous, coherent or more powdery sediment. Occasional cultures grew with formation of granules which sedimented leaving the supernate more or less clear

Suspensions in saline, again with occasional exceptions, were remarkably stable and often did not sediment until after several days in the refrigerator In the agglutination tests there was hardly any sedimentation in the control tubes within 20–24 h.

Other physiological and biochemical characters of the strains are presented in Tables 1 3 The results show that the strains are remarkably uniform. Out of 41 characters listed, 33 were identical, 18 positive and 15 negative and only 8 showed variable results. Most of these reactions are probably inherently variable Thus 4 were delayed respectively negative fermentation reactions, the results of which might vary in repeated tests. The methyl red test is notoriously unreliable and the terminal pH of these strains is close to the turning point of methyl red. Differences in growth at room temperature may be

TABLE 1 *Alpha Haemolytic Streptococci with Spreading Colonies. Common Positive Characters*

Small diplococci and short chains.
Greening and haemolysis on blood agar
even turbidity in Todd Hewitt broth.
Stable in saline suspension.
Polysaccharide in 5 per cent sucrose broth.
Acid from glucose galactose laevulose, mannose lactose maltose, sucrose trehalose, salicin.
pH in glucose broth (4.5–4.6)
Precipitation and agglutination in sera against strains 13843 and 14558.
Precipitation in group H serum

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TABLE 2. *Alpha Haemolytic Streptococci with Spreading Colonies Common Negative Characters*

Mucoid colonies on 5 per cent sucrose agar
Acid from mannitol, dulcitol, arabinose.
Growth with 10 per cent bile,
40 per cent bile, 4 per cent NaCl
6.5 per cent NaCl, after heating to
60 C for 30 min.
Indol, growth in nitrate broth.
Optochin sensitivity Catalase.
Production of Acetoin.
Splitting of hippuric acid.

due to minor temperature variations, and differences in bacitracin sensitivity were actually negligible. The only difference which appeared to be stable was the results of aesculin splitting as shown by repeated tests.

Thus the strains constitute a very homogeneous group with reaction patterns similar

TABLE 3. *Alpha Haemolytic Streptococci with Spreading Colonies. Variable Characters*

Acid from glycerol (18– 1 w+) raffinose (10 9–) sorbitol (5d*+ 14–) inulin (13d+ 6–), aesculin (17+ 2–) methyl red (17+ 1± 1–), growth at room temperature, 20-22 C, (9+ 10–) bacitracin sensitivity (15 tr+ 4–)

* d = delayed reaction w+ = weak reaction, tr+ = very narrow zones of inhibition.

to those reported for group H streptococci (*S. sanguis*) (1 2, 3 12, 16) The two reference strains agreed with the other strains in the reactions that were tested. All strains were strongly to moderate sensitive to penicillin.

The reactions in 5 per cent sucrose broth varied from one culture to the next, some cultures turning into firm jelly others showing smaller lumps of jelley and others again

TABLE 4 *Agglutination Reactions of Alpha Haemolytic Streptococci with Spreading Colonies*

Antigen for agglutination and absorption	Immune sera: No. 1 – anti 13843, Unabsorbed	No. 1 – anti 13843, Absorbed I*	No. 1 – anti 13843, Absorbed II**	No. 2 – anti-14558, Unabsorbed	No. 2 – anti-14558, Absorbed I	No. 2 – anti-14558, Absorbed II**
13843	*1280*			160	<40	1280
14241	1280	160	1280	320	<40	1280
14496	640	320	1280	1280	320	320
14502	640	40	1280	640	40	640
14506	2560	160	1280	1280	320	320
14558	640	40	1280	*1280*		
14567	640	160	1280	640	40	1280
19	1280	160	1280	1280	160	160
34	2560	80	80	320	40	1280
419	1280	80	1280	1280	160	160
445	1 80	40	1280	1280	80	1280
480	640	40	1280	1280	40	160
72?	80	<40	1280	1280	160	160
736	2560	<40	1280	640	<40	1280
2908	640	80	1280	640	40	1280
2912	640	<40	1280	320	40	1280
2934	640	40	1280	1280	160	160
2936	640	160	1280	1280		
2974	1280	320	1280	1280	40	1280
NCTC 7863	2560	80	1280	1280	<40	1280
NCTC 7865	1280	<40	1280	640	<40	1280

I = agglutination with absorbing strain.
**; II = agglutination with homologous strain.

only showing slight increase of viscosity or reactions might be absent. Sixteen of the strains, and the only reference strain that was tested, showed production of jelly in one or more cultures, the rest showed production of polysaccharide after addition of ethanol to the cultures.

Serology

All strains were found to have a common precipitinogen, probably a group specific polysaccharide. Extracts of all strains, including the two reference strains (but not including the 6 non-spreading strains) produced comparable, fairly large quantities of precipitate with each of the four immune sera against two strains (13843 and 14558). In order to identify this antigen, tests with commercial sera against the known sero-groups were carried out. The first tests with sera that had been in use for some time and which only sufficed for testing a few strains, showed in group H serum some faint, questionable precipitations with some strains. For this reason the tests were repeated using a new set of lyophilized commercial sera against groups A to Q. The sera were used on the day that they were redissolved. All tests were negative. Finally repeated tests were done with grouping sera against groups A to S from another manufacturer. Now all extracts of the spreading strains as well as of the two reference strains gave distinct, although not very strong precipitation with the group H serum. The results are taken to indicate that these strains do belong to group H.

As shown in Table 4 all strains were agglutinated by one of the immune sera against each of the two strains.

Most titres were close to the homologous ones, from one dilution step above to one below these titres. In one serum all strains except one showed titres within this range, and in the second serum, only 4 strains gave titres 4 to 8-fold lower than the homologous strain.

Absorption tests showed that the agglutinogens are heterogeneous, as in one serum, only one strain removed the agglutinins against the homologous antigen, and in the second serum, 7 strains caused a significant reduction of the homologous titre. Thus several serotypes may be represented in the collection. Although the two reference strains were agglutinated to high titres, they failed to remove the homologous agglutinins.

DISCUSSION

The results show that the streptococcal strains selected on account of their production of spreading zones on blood agar constitute a very homogeneous group with characters corresponding to those reported for group H strains, or *S. sanguis* (1 2, 3 12 15) except in minor details which may not be significant.

All strains had a common precipitinogen, apparently identical with the group specific antigen of group H, and consequently can be classified as *S. sanguis*. The results suggest that the production of spreading colonies due to twitching and the possession of fimbriae may be a common, possibly an exclusive property of this taxon. If extended studies confirm that this is true, these characters would gain taxonomic interest and might be useful in isolating and identifying these streptococci. It is necessary however to investigate how common and how stable these characters are in *S. sanguis* and whether they only occur in this taxon. In this connection it is of some interest that one of the two reference strains failed to produce spreading colonies.

Neither twitching motility nor possession of polar fimbriae in gram-positive bacteria has, to our knowledge, been described before. This new observation, therefore, confirms and markedly extends the correlation already shown to exist between twitching motility and polar fimbriation in gram-negative bacteria (5 6 7).

Peritrichously arranged fimbriae have been demonstrated in only one species of gram-positive bacteria, viz. *Corynebacterium renale* (16). It is true that *Swanson et al.* (13) and *Swanson & McCarty* (14) refer to the M antigen surface layer of streptococci as fimbriae, but they fail to give further details and the described material does not have the regular appearance of fimbriae. To us it looks more like capsular material or "slime threads"

Spreading growth of streptococci has been described before, but was shown to be due to sliding (4) In a collection of 25 strains of streptococci with this kind of surface translocation there were strains of *S mitleri S mitior S salivarius* and strains which could not be assigned to any known, well-defined taxon. These strains had been isolated from a variety of clinical specimens (*Henrichsen J.*, unpublished observations)

The serological studies show that the strains used in the present investigation have one precipitinogen in common as well as one agglutinogen, but absorption tests also reveal heterogeneous agglutinogens. The explanation may be that the common agglutinogen may be identical with the group-specific antigen and that the heterogeneous agglutinogens may be type-specific and of a different nature. The role of group-specific antigens of streptococci as agglutinogens has apparently not been much studied. But there is some evidence (10) that, at least in group A streptococci, the C-polysaccharide may act as an agglutinogen.

The possible association of fimbriation in streptococci with other biological properties such as haemagglutination, adhesiveness, and competence of transformation deserves investigation. Adhesiveness of streptococci—often ascribed to production of glycans—has been much discussed in relation to dental disease. Adhesiveness due to fimbriae might also be of importance in this connection as well as in relation to subacute bacterial endocarditis where *S sanguis* is known to play a considerable role. As for transformation, it is well known that strains of *S sanguis* have shown competence, and—particularly the strain "Challis"—have been much used as recipients.

Work is in progress to study some of these problems.

REFERENCES

1 *Colman C & Williams R E. O* Taxonomy of some human viridans streptococci. In Wannamaker L. W & Matsen, J M. (Eds.) Streptococci and streptococcal disease Academic Press, New York & London 1972, pp. 281–299

2 *Cowan S T & Steel K. J* Manual for the identification of medical bacteria. Cambridge University Press, 1965

3 *Dodd R. L.*, Serological relationship between group H and *Streptococcus sanguis*. Proc. Soc. Exper Biol. Med. *70* 598–599 1949

4 *Henrichsen J* Bacterial surface translocation A survey and a classification. Bact. Rev *36*: 478–503 1972.

5 *Henrichsen J & Blom J* Correlation between twitching motility and possession of polar fimbriae in *Acinetobacter calcoaceticus* Acta path. microbiol. scand. Sect. B, *83* 103–115 1975

6. *Henrichsen J & Blom J* Examination of fimbriation of some gram-negative rods with and without twitching and gliding motility. Acta path. microbiol. scand. Sect. B, *83* 161 170 1975.

7 *Henrichsen J Froholm L. O & Bøvre, K.* Studies on bacterial surface translocation. 2 Correlation of twitching motility and fimbriation in colony variants of *Moraxella nonliquefaciens M bovis* and *M kingii*. Acta path microbiol. scand. Sect. B, *80* 445–452, 1972

8. *Henriksen S D* Corroding bacteria from the respiratory tract. I. *Moraxella kingii*. Acta path. microbiol. scand. *75* 85–90, 1969.

9 *Henriksen S D & Bøvre K.* Corroding and spreading colonies in *Moraxella nonliquefaciens*. Acta path. microbiol. scand. *76* 459–463 1969

10. *Henriksen S D & Heidelberger M* Chemical studies on bacterial agglutination. VI. The agglutinin content of antisera to hemolytic streptococci J exp Med. *74* 105–114 1941.

11 *Pedersen, K B. Froholm L. O & Bøvre K.* Fimbriation and colony type in *Moraxella bovis* in relation to conjunctival colonization and development of keratoconjunctivitis in cattle. Acta path. microbiol. scand. Sect. B, *80* 911–918, 1972.

12. *Porterfield R S* Classification of the streptococci of subacute bacterial endocarditis J. gen Microbiol *4* 92–101 1950.

13 *Swanson J Hsu K. C & Gotschlich, E C* Electron microscopic studies on streptococci I M antigen. J exp. Med. *130* 1063–1091, 1969

14 *Swanson, J & McCarty M* Electron microscopic studies on opaque colony variants of group A streptococci. J Bact. *100* 505–511 1969

15 *White J C & Niven C F* Streptococcus S.B.E. A streptococcus associated with subacute bacterial endocarditis J Bact. *51*: 717 722, 1946

16 *Yanagawa R., Otsuki, K & Tokui T.*: Electron microscopy of fine structure of *Corynebacterium renale* with special reference to pili. Jap. J Vet. Res. *16*: 31–38, 1968.

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EFFECT OF β PROPIOLACTONE ON INFECTIVITY AND HAEMAGGLUTININ OF THE BK VIRUS

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Pitko, V.-M., Pyökari, P., Näse, L. & Mäntyjärvi, R. Effect of β-propiolactone on infectivity and haemagglutinin of the BK virus. Acta path. microbiol. scand. Sect. B 83 141–144 1975.

The effect of β-propiolactone (BPL) on the infectivity and haemagglutinating properties of BK virus was studied. No virus multiplication was observed when Vero cell cultures were inoculated with virus treated with 0.1 per cent or higher concentration of BPL. On the other hand, treatment of BK virus with 0.1 per cent or lower concentration of BPL had no apparent effect on viral haemagglutinin. BPL at a concentration of 0.1 per cent could therefore be used to prepare BK virus haemagglutinin which contains little or no infectious virus. Inactivated haemagglutinin seems to be somewhat labile against freezing and thawing, but storage at 4 C had no effect on it. Identical haemagglutination inhibiting antibody titres were obtained when human sera were tested with standard haemagglutinin or with haemagglutinin inactivated with BPL.

Key words BK virus infectivity haemagglutinating properties β-propiolactone.

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A new human virus, designated the BK virus, has recently been isolated from renal transplant patients (1). This virus belongs to the SV 40-polyoma subgroup of papovaviruses, and it is antigenically related to SV 40 virus and to JC virus, another new human papovavirus (9). Little is known of the clinical symptoms caused by BK virus infection. It seems to be a common virus: 70 to 90 per cent of children acquire antibodies to it before school age as reported from England, Finland and the U.S. (2, 6, 13). This primary infection probably goes unnoticed in most cases (6), but in the same way as the JC virus has been connected with progressive multifocal leuco-encephalopathy it is conceivable that BK virus infection could also take another more serious form.

BK virus agglutinates human and guinea pig erythrocytes (5) and haemagglutination inhibition offers a convenient method by which to measure antibodies to BK virus. In the routine laboratory it would be very desirable to use non-infectious reagents in serological tests especially in work with viruses of unknown potential. β-propiolactone (BPL) has been found useful in inactivating the infectivity of several viruses while leaving the haemagglutinating properties intact (4, 12, 14). The present paper describes the results of our experiments with a view to examining the possibility of preparing non infectious BK virus haemagglutinin by treatment with BPL.

MATERIAL AND METHODS

Cell lines and virus. Vero cells were used to propagate BK virus strain obtained from Dr *Sylvia Gardner*. Infected cells were maintained in BME diploid medium (Gibco Grand Island, N Y) with 0.2 per cent bovine serum albumin, fraction V (Armour Pharmaceutical Co., Eastbourne, England) and 1 0 mM arginine. Haemagglutinin was extracted from infected cells as described earlier (7)

Infectivity titration. End point dilution method was used. Vero cells were grown in wells of plastic plates (Micro Test II Falcon plastics, Oxnard, Ca.) Appropriate ten-fold dilutions of virus were made in maintenance medium, and 0.2 ml volumes of virus inoculated on cells. Plates were incubated in 5 per cent CO_2 atmosphere, and the maintenance medium was changed every 10 to 14 days. Growth of the virus was detected by haemagglutination as follows. Plates were sealed with adhesive tape, and frozen and thawed to detach and to break the cells. To each well, 0.05 ml of an 0.5 per cent suspension of human type 0 erythrocytes was added. Erythrocyte suspension was made in 0.05 M phosphate buffered saline pH 7 0 containing 0.01 per cent gelatin. Erythrocytes were allowed to settle, keeping the plates in a tilted position on ice slurry Haemagglutination was read after 1 to 1.5 hours incubation.

Haemagglutination and haemagglutination inhibition These tests were done as described earlier (6) To remove nonspecific HI inhibitors, sera were treated with sodium periodate (11) instead of receptor destroying enzyme used in earlier tests.

β-propiolactone (BPL) treatment BPL (Fluka AG, Buchs SG Switzerland) was stored at −20 C. BK virus was treated with BPL by the method of *Neff & Enders* (8) A bicarbonate diluent was used to make a 1 per cent solution of BPL (1 68 gm of $NaHCO_3$ in isotonic saline) An appropriate amount of 1 per cent BPL was added to virus to give the wanted, final concentration The mixture was shaken at 4 C for 10 minutes, and then incubated at 37 C for two hours After overnight incubation at 4 C to assure hydrolysis of BPL, viruses were tested for infectivity and for haemagglutination Viruses were then stored at 4 C.

RESULTS

Infectivity titration Because of difficulties involved in the evaluation of the cytopathic effect (CPE) of BK virus in Vero cells, experiments were made to adapt HA reaction to detect multiplication of virus in titration plates. A seed virus was titrated in two separate experiments. Four wells were inoculated with each virus dilution. After proper incubation, virus-containing wells were identified with HA and the infectivity titre of the seed virus was calculated as 50 per cent tissue culture infectious dose ($TCID_{50}$) (10) The results showed that titres in the two experiments were, at a given time after titration, within 0.5 log units. In the same experiment the effect of incubation time on titres was also studied. Using replicate titration plates, the infectivity titre of the virus was determined after three four and five weeks of incubation, and it was found to increase by about 0.5 log units during this period. The infectivity titration procedure described under Methods is the result of these experiments.

Effect of BPL on infectivity In a preliminary test, no virus multiplication was detected in Vero cell culture tubes after inoculation with BK virus treated with 0.1 per cent of BPL. Viruses treated with 0.2 per cent or 0 4 per cent of BPL were toxic to cells if inoculated undiluted. In another experiment, BK virus was treated with four different concentrations of BPL, and titrated for infectivity as described under Methods. Results are

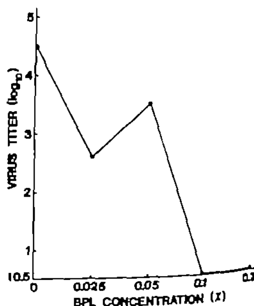


Fig 1 Effect of BPL on the infectivity of BK virus. Virus was treated with various concentrations of BPL, and titrated for infectivity in Vero cells. Infectivity titres are expressed as $TCID_{50}$/0 2 ml.

shown in Fig 1 After treatment with 0.05 per cent of BPL, about 10 per cent infectivity was still present whereas no infectious virus was detected in preparations treated with higher concentrations of BPL. Again, viruses treated with 0.1 per cent or 0.2 per cent of BPL were toxic to Vero cells if inoculated undiluted, and therefore only the upper limit of infectivity of $10^{0.5}$ $TCID_{50}$ per 0.2 ml can be ascribed to them.

Effect of BPL on haemagglutinin The effect of BPL on HA activity of BK virus was tested in three experiments. The results are shown in Fig 2 BPL concentration of 0 1 per cent seems to be the limit above which BPL begins to decrease the HA titre of virus. HA titrations shown in Fig 2 were performed immediately after BPL treatment. Storage of BPL-treated virus in frozen form appeared to have a deleterious effect on haemagglutinin. In further tests it was found that a cycle of freezing and thawing decreased the titre of virus treated with 0.2 per cent of BPL by 2 to 3 $\log_2$ units. In addition, the agglutination pattern near the endpoint was granular and agglutination was weak, as indicated by disrupture of the agglutinate when the plate was handled A similar effect, although less marked, was observed when virus treated with 0 1 per cent of BPL was frozen and thawed. Repeated cycles of freezing and thawing evoked further decrease in the titres. In contrast to this, 6 weeks storage at 4 C had no effect on the HA titre of virus treated with 0.2 per cent or lower concentration of BPL.

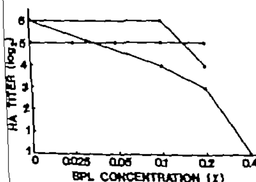


Fig 2 Effect of BPL on the haemagglutinin of BK virus. Virus was treated with various concentrations of BPL in three separate experiments and titrated for haemagglutinin.

BPL-treated haemagglutinin was next tested for use in HI test. Ten human sera with varying HI antibody titres against BK virus were titrated using standard haemagglutinin, haemagglutinin treated with 0 1 per cent concentration of BPL, and haemagglutinin which had been sham-treated with bicarbonate. Identical titres were obtained with all three haemagglutinins.

DISCUSSION

Viral reagents used in serology often contain infectious virus and thus constitute a hazard in the laboratory Replacement of these reagents by non infectious ones would require the inactivation of the infectivity without affecting antigenic or biological properties of the virus.

BPL is an alkylating agent with a strong antimicrobial power It is used in virology and cell biology to inactivate Sendai virus for cell hybridization experiments (8) In a more detailed study on the effect of BPL, five biological properties of Sendai virus *viz.* infectivity haemagglutination, haemolysis, cell fusion and neuraminidase were dissociated on the basis of sensitivity to BPL (14) Haemagglutinin was found to be the activity most resistant to BPL. Therefore BPL treatment seems to offer a way of preparing non-infectious viral haemagglutinins.

In the experiments described in this paper we have tested the effect of BPL on infectivity and on the haemagglutinin of BK virus. Initially difficulties were found to be involved in infectivity titrations based on recording of CPE. HA reaction was therefore adapted to detect multiplication of virus in infectivity titrations. Initial difficulties involved in the recording of haemagglutination were eliminated when gelatin was added to erythrocyte suspension (5) If titration plates are kept at an angle of about 30 during the settling of erythrocytes, a semicircular band of non-agglutinated erythrocytes is clearly

visible on the side of the wells not containing virus. It is advisable to leave a few wells in each plate uninfected to serve as negative controls for haemagglutination. At first we confirmed the macroscopic reading by taking samples from wells on a microscopic slide and recording the agglutination under a microscope. This, however was found to be unnecessary because the same results were obtained by both methods.

As could be expected, infectivity of BK virus was inactivated with BPL when the latter was used in appropriate concentrations. However a concentration of 0 1 per cent of BPL was needed to render BK virus noninfectious, whereas a concentration of 0 01 per cent has been found sufficient to destroy the infectivity of Sendai (14) and rabies (4) virus. A concentration of 0 1 per cent of BPL has been reported earlier to be suitable for the preparation of non infectious haemagglutinins of several viruses, including adenoviruses (12) The range of BPL concentration sufficiently high to inactivate BK virus infectivity without interfering with HA was narrow The concentration of 0 1 per cent, however apparently left the haemagglutinin intact, whereas no virus multiplication was detected in Vero cells infected with the inactivated virus.

Two more requirements have to be fulfilled before an inactivated viral haemagglutinin is suitable for the routine used viz (i) stability at storage and (ii) identical reactivity with standard haemagglutinin in HI tests. BK virus haemagglutinin treated with 0 1 per cent BPL and stored at 4 C seems to possess both properties.

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REFERENCES

1 *Coleman D V Gardner S D & Field A M* Human polyomavirus infection in renal allograft recipients. Brit. Med. J 3 371–373, 1973
2. *Gardner S D* Prevalence in England of antibody to human polyomavirus (B K.) Brit. Med. J *1* 77–78, 1973.
3 *Gardner S D Field A M Coleman D. V & Hulme B.* New human papovavirus (B K) isolated from urine after renal transplantation. Lancet *1* 1253–1257 1971
4 *Halonen P E., Murphy F A Fields, B N & Reese D R.* Hemagglutinin of rabies and some other bullet-shaped viruses. Proc. Soc. Exp. Biol. Med. *127* 1037–1042, 1968.
5 *Inouye S* Nonspecific adsorption of proteins to microplates. Appl. Microbiol. 25 279-283, 1973
6 *Mäntyjärvi R. A., Meurman, O H Vihma, L. & Berglund B* A human papovavirus (B.K.) biological properties and seroepidemiology Ann. Clin. Res. *5* 283–287 1973.
7 *Mäntyjärvi R A. Arstila, P P & Meurman, O H.* Hemagglutination by BK virus, a tentative new member of the papovavirus group. Infect. and Immunity 6 824–828, 1972.
8. *Neff J M & Enders J F* Poliovirus replication and cytopathogenicity in monolayer hamster cell cultures fused with beta-propiolactone-inactivated Sendai virus Proc. Soc. Exp Biol. Med. *127* 260–267 1968.
9 *Penney J B Jr & Narayan O* Studies of the antigenic relationships of the new human papovaviruses by electron microscopy agglutination. Infect. and Immunity *8* 299-302, 1973
10. *Reed L. J & Muench H* A simple method of estimating fifty per cent endpoints. Amer J Hyg. *27* 493–497 1938.
11 *Robinson R Q. & Dowdle W R* Influenza viruses. In Lennette E. J & Schmidt, N. J (Eds.) Diagnostic procedures for viral and rickettsial infections, 4th ed. American Public Health Association, Inc., New York 1969, pp 428–429
12. *Sever J L. Castellano G A. Pelon, W Huebner R. J & Wolman F* Inactivation of the infectivity of viral hemagglutinating antigens with the use of betaprone. J Lab Clin Med. *64* 983–988, 1964
13 *Shah K V Daniel R. W & Warszawski, R M* High prevalence of antibodies to BK virus, an SV40-related papovavirus, in residents of Maryland. J Infect. Dis. *128* 784-787 1973
14 *Waisberg M A Hjorth R N & Hare C.* Effect of β-propiolactone on Sendai virus. Appl Microbiol. 22 618–621 1971

a path. microbiol. scand. Sect. B, 83 145–152, 1975

NEW IMMUNOFLUORESCENCE METHOD FOR THE IDENTIFICATION OF GROUP A, B, C E, F, AND G STREPTOCOCCI

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Cars, O Forsum, U & Hjelm, E. New immunofluorescence method for the identification of group A, B C, E, F and G streptococci. Acta path. microbiol. scand. Sect. B, 83 145–152, 1975

An immunofluorescence method for the identification of groups A, B, C, E, F and G beta-haemolytic streptococci has been developed, using FITC-labelled $F(ab')_2$ fragments of IgG with optimal staining characteristics. The method is useful for the rapid confirmation of beta-haemolytic streptococci in cultures in a blind comparison with Lancefield grouping, there was a 97 per cent agreement.

Key words Streptococci group identification immunofluorescence.

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The importance of non-group-A streptococcal infections in man has recently attracted considerable interest. The streptococcal groups now recognized as those most often associated with human disease are A, B, C, and G (1 4 15 26, 49) Therefore, the clinicians' demands on the clinical microbiologist to identify precisely betahaemolytic streptococci are increasing. Factors determining the choice of tests to be used for grouping are accuracy rapidity and cost. The Lancefield technique (35) and several modifications of the latter (8, 12, 22, 47) are by now firmly established because of their reliability however the need for pure culture of the organism to be tested and extraction of the group polysaccharide from the cell-wall makes these techniques time-consuming and costly Serological tests by which to identify antigens directly on the intact streptococcus i.e. immunofluorescence agglutination and co-agglutination (2, 5 21 37 40) eliminate these procedures and are therefore potentially more rapid. Immunofluorescence identification of group A streptococci is now a standard method in several laboratories (2 11 13 49) reports on other major non-group-A streptococci are few however and details are lacking (37 43 45)

A defined immunofluorescence technique using $F(ab')_2$ fragments of IgG has been developed at our laboratory It takes advantage of the fact that troublesome cross-reactions with *staphylococcal* protein A does not occur if $F(ab')_2$ fragments (*16*) are used and it has been successfully applied for the identification of several bacterial species (10, 17–19 23 25) The use of $F(ab')_2$-fragments of *IgG* also excludes nonspecific reactions between normal rabbit IgG and streptococci group A, C, and G (6, 9 30) An immunofluorescence method based on $F(ab')_2$-fragments thus seems to be an important step

towards the standardization of reagents for the identification of betahaemolytic streptococci.

MATERIAL AND METHODS

Strains

The reference strains were kindly supplied as follows

Collections of *Streptococcus pyogenes* by *Rebecca Lancefield* Rockefeller University New York, and *J ri Rotta*, WHO Streptococcus Typing Reference Center Prague.

S pyogenes group A 71-69 T4/95/RB5/ by Dr *D Johnson* Department of Pediatrics, University of Minnesota, Minneapolis.

S pyogenes group G 867A by Dr *R Facklam* CDC, Atlanta.

S pyogenes group F UI 103 by Statens veterinärmedicinska anstalt, Stockholm.

In addition the following strains isolated at the routine laboratory of the Microbiology Institute Uppsala, were used

For specificity testing *St phylococcus aureus* Cowan I representative strains of *Streptococcus sanguis mitis Neisseria gonorrhoea, meningitidis flava, St phylococcus albus* and *Pneumococcus* among them Colindale type 14

With a view to comparison between Lancefield grouping and immunofluorescence, strains of beta haemolytic streptococci were used.

Culture

All strains were cultivated on solid, sheep blood agar or on haematin agar for 18 h at 37 C in 5 per cent CO_2 before testing.

Immunization

Antigen for group A, C, and G betahaemolytic streptococci was prepared essentially according to *Krause et al.* (29) by culture of appropriat strains in Todd-Hewitt broth for 48 h at 37 C. The pH was adjusted to 2.0–2.5 by addition of HCl the culture was left at room temperature for 12 h and then neutralized by adding 1 M NaOH, followed by washing three times in 0 15 M N Cl. The pH was again lowered to 2 pepsin was added to 0 1 per cent and the cell suspension was incubated for 2 h at 37 C, followed by neutralization and washing twice in 0 15 M NaCl The bacterial suspension was resuspended to pprox. 10^9 bacteria/ml in 0.15 M NaCl a d kept frozen at –20 C until used. Acid heat extrac s from each suspension were prepared and tested for specificity against commercial, group-specific ant era (Wellcome) in capillary tube precipitation

White rabbits were injected intravenously every other day for 4 weeks 0.5 ml per injection during the first week and 1.0 ml during the remaining 3 weeks. One week after the last injection, the animals were test bled and sera showing indirect immunofluorescence titres, using FITC labelled antirabbit IgG Behringwerke AG of 1/40–1/80 with homologous strains as antigen, were collected

Groups B E, and F streptococci were prepared and used for immunization in the same way except that the HCl and pepsin steps were omitted. The rabbits were bled one week after the last injection and sera showing indirect immunofluorescence titre, using FITC labelled antirabbit IgG Behringwerke AG of 1/40–1/80 with homologous strains as antigen, were collected.

Preparation of FITC-labelled F(ab) -fragments of IgG

After isolation of the IgG fraction (14) from antisera to *Streptococcus pyogenes*, $F(ab')_2$-fragments of IgG were prepared by pepsin digestion using 1 mg of enzyme per 50 mg of IgG in 0.1 M sodium acetate (pH 3 0) for 7 h. The $F(ab')_2$ fragments were conjugated with FITC (BDH biochemicals Poole England) using 30 μg of dye per mg of protein under vigorous stirring at room temperature for 1 h (pH 9.5) and fractionated on Sephadex G-25 equilibrated with 0.0175 M Na_2HPO_4 (pH 4 7) into fractions with different F/P ratios. The first peak to emerge from the column was collected and its pH was adjusted to 7.2. Conjugated $F(ab)_2$ fragments with higher F/P ratios, which showed firmer absorption to the gel and therefore eluted in second peak, were discarded (18) The F/P ratio was calculated according to *Forsum* (18)

Dilution and Absorption of Conjugates

The conjugates were diluted in phosphate-buffered saline (PBS) (pH 7 2) and the highest dilution giving 3+ staining of the homologous organism was recorded as the specific titre of each conjugate.

Absorption of conjugates was performed using two volumes of conjugated F(ab) fragments and one volume of packed, heat-killed cells of the heterologous, cross-reacting organism t 37° C for 1 h. These cells were taken from an 18-hour-broth culture, heat killed at 56 C for 30 min and then washed 3 times with PBS. Conjugates were considered satisfactory if a reduction in specific titre did not occur while the reaction with the strain used for absorption was abolished. Group F conjugates, however were rendered group-specific only after adsorption that reduced the working titre of the conjugates by one or two d lutions.

taining of Smears and Recording Procedures

Smears were prepared by dipping a platinum loop into a colony and applying the bacteria directly onto slides in one drop of PBS. The smears were allowed to air-dry and were then gently heat fixed. One drop of conjugate was placed on a smear and incubated for 20 min at 37 C. The smears were washed with PBS for 15 min and mounted under a cover slip with phosphate-buffered glycerine (pH 7.2)

The preparations were read under a Leitz Orthoplan fluorescence microscope equipped with incident light, Osram HBO 200 mercury lamp and a KP 490 as primary filter and a TK510/K515 as the secondary. All observations were made under × 450 magnification. The following criteria were used for recording the specific fluorescent staining of microorganisms: 3+ indicating intensely fluorescent margins, well-marked edges 2+ indicating faintly fluorescent margins, edges usually well-marked 1+ indicating barely distinguishable fluorescent margins with diffuse edges.

Lancefield Grouping

Lancefield grouping was performed according to Fuller (22) using commercial sera (Wellcome)

RESULTS

haracterization of FITC-labelled nti A B C E F and G Streptococcal (ab) Fragments of IgG

The conjugated and fractionated antistreptococcus $F(ab)_2$ fragments were characterized by their specific and non-specific staining in immunofluorescence. Conjugates (7 mg/ml) with F/P ratio (µg/mg) of approximately 5–10 were optimal in this system. No staining occurred if *S. aureus* Cowan I was used. A total of 31 rabbits were immunized, yielding specific titres expressed as a 3+ reaction of 1/2 to 1/20 A titre of 1/8 was most frequently observed.

Cross-reactions

In preliminary experiments, a collection of reference strains were used for immunization. Sera giving the best specific titres during screening with indirect immunofluorescence were fractionated, pepsin-digested, conjugated, and then thoroughly studied. The strains selected from these preliminary experiments by which to produce group-specific conjugates are listed in Table 1 Table 2 shows the cross-reactions of the unabsorbed conjugates at the highest dilution still giving a 3+ reaction. It will be noted that the most prominent cross-reactions are those within the B group and those between anti-A conjugate and C cells. Several of the conjugates not used in the final set were unsuitable for the production of group-specific conjugates due to strong cross-reactions or low titres It is remarkable that several anti-group-A conjugates, i.e. anti A8143 cross-reacted heavily

TABLE 1. *Strains Used for Immunization and Absorption of F(ab)₂-fragment Conjugates for the Grouping of Betahaemolytic Streptococci in Immunofluorescence*

Group	Strain used for: Immunization	Absorption
A	71-679-T4/95/RB5 [1]	C6212 [5]
B	090R(1965) 5/19/70 [2]	—
	Ia-090-2/4/71	—
	Ib-H36B-10/2/68	—
	Ic A909-5/19/70	—
	II 18RS21/19/3-9/23/71 [2]	—
	III-D136C-6/2/67	—
C	C-74-1/15/71 [3]	71-679-T4/95/RB5 [1] F 105
E	K131 [3]	—
F	H60R	68-7014
G	867A	A8143 [4] C6212 [5]

Strains received from: Johnson, Lancefield [2] Rotta, [3] Facklam, [4] Own collection, SVA.

TABLE 2. *Cross-reaction Pattern in Immunofluorescence at Highest Dilution Giving 3+ Reaction of the Strain Selected for the Production of Antisera to Lancefield Groups of Betahaemolytic Streptococci*

FITC-labelled $F(ab')_2$-fragments of IgG to groups	Antigen betahaemolytic streptococci groups										
	A	B_{090R}	B_{Ia}	B_{Ib}	B_{I}	B_{II}	B_{III}	C	E	F	G
A	3+										
B_{090R}	1+	3+	1+			2+	1+				
Ia			3+	1+	1+						
Ib				3+	1+						
I		1+	1+	2+	3+	2+	2+				
II						3+					
III							3+				
C	2+							3+	1+	1+	
E									3+		
F										3+	
G								1+			3+

Strains used for immunization listed in Table 1

with group C and G cells. A *strong* cross-reaction between anti-group F conjugate (F-68C) and group G strains was also noted, but anti (F H60R) conjugate showed no such cross-reaction. No cross-reactions have been observed with group D streptococci.

Reaction Patterns of the Finally Absorbed Conjugates or Pool Conjugates

The anti-groups A, B, C, E, F and G conjugates chosen for further studies were subjected to cross-absorption with a wide range of appropriate heterologous strains. The absorptions chosen for routine work are tabulated in Table 1 and the reactions of the final set of group-specific conjugates are seen in Table 3.

The criteria for acceptable absorption included retained specific staining at 3+ level, a titre giving a clear-cut difference between staining of homologous strains and cross-reacting strains. Group B was best studied if a pool of the different anti-B conjugates was used.

All absorbed or pooled conjugates listed in Table 2 were tested for cross-reactions with other cocci. No cross-reactions with strains of *Staphylococcus albus Streptococcus viridans, Neisseria gonorrhoeae flava* and *meningitidis*

TABLE 3. *Reactions in Immunofluorescence of FITC-labelled F(ab')-conjugates to Selected Groups of Betahaemolytic Streptococci after Absorption or Pooling of Group-specific Conjugates*

Absorbed or pooled conjugates to Lancefield groups	Antigen*										
	A	B	B_{Ia}	B_{Ib}	B_{I}	B_{II}	B_{III}	C	E	F	G
A	3+										
B_{Pool}		3+	3+	3+	3+	3+	3+				
C	1+							3+			
E									3+		
F										3+	
G											3+

* Strains used for immunization listed in Table 1

TABLE 4 *Comparison between Capillary Tube Precipitation Test Using Fuller Antigen and Immunofluorescence Using FITC-labelled F(ab')₂-fragments for Grouping of Betahaemolytic Streptococci*

Total number of strains grouped according to Lancefield technique		Number of strains shows 3 + immunofluorescence reaction with groupspecific conjugates to groups					
		A	B	C	F	G	other
A	43	41					
B	42		41				
C	33			33			
F	2				2		
G	58	2*				58	
other	16		1				15

Two strains showed immunofluorescence reaction with both antigroup A and G conjugates.

and *Pneumococcus* were observed, with the exception of a strong cross-reaction between anti-B III conjugate and one strain of pneumococcus type 14. All available pneumococcal *strains were re-tested with anti-B III* conjugate and any further cross-reactions were not observed.

A blind comparison between the tube precipitation with Fuller antigen and the developed immunofluorescence technique was made using 170 consecutive strains sent to the typing laboratory. All strains were tested with all conjugates. It appears from Table 4 that, although it is most probable that antigens other than group-specific polysaccharide take part in the immunofluorescence reaction, agreement between the two methods was found to be very good. Trypsin treatment of group A, C, and G cells did not improve the staining of these cells. Three false negative strains occurred in immunofluorescence and two could not be grouped, i.e. the two tests agreed in 97 per cent. During the period in which the comparison was made no group F strains were identified by precipitation but one was grouped as E by immunofluorescence.

DISCUSSION

It is well documented that immunofluorescence is a rapid and simple technique by which to detect and type bacteria in pure cultures as well as on direct smears in certain applications (2, 10 19 24 25). Increasing attention has been paid to a standardization of immunofluorescence as regards the criteria of preparation of conjugates and quality control of the conjugates defining their specific and non-specific staining properties (3). According to recent reviews, however the standardization of reagents by which betahaemolytic streptococci may be typed is not considered a thoroughly investigated field which should be given the highest priority (3). In recent publications from this laboratory an immunofluorescence method based on FITC-labelled F(ab')₂ fragments with defined characteristics has been described and applied to different bacterial species (10 17–19 23, 25). Distinguishing features are the lack of cross-reactions with protein A containing *S. aureus* (16) and the simplicity with which the defined reagents for direct immunofluorescence are produced.

Application of the method *for the grouping* of betahaemolytic streptococci exposed, as expected specific problems: conjugate titres. *Krause et al.* (29) showed in extensive studies that rabbits can be divided into genetically different groups: one in which *response is* high and one in which response to streptococcal group-specific carbohydrate is low. As expected from Krause's work, rather low conjugate titres in several conjugates were recorded; however, our final *conjugates had* specific staining titres which differed significantly from those obtained by staining of

cross-reacting strains if adequate strains for antigen production were used. Others have reported very high titres (43) a finding which may be explained either because different criteria were used for specific staining or the choice of rabbits may have been different.

Cross-reactions and non-specific reactions between group A, C, and G streptococci are well known in immunofluorescence (6 7 9 20 21 27 28 30 36 37) In addition, *Kronvall* (30) and *Christensen & Kronvall* (6) have shown that groups A, B, C, and D streptococci react with rabbit and human IgG irrespective of their antibody combining sites. No such reactivity was found if F(ab)$_2$-fragments of IgG were used a fact that further shows the theoretical and practical advantage of using F(ab)$_2$-fragments. Cross-reactions other than those between group A, C, and G have also been observed in immunofluorescence (21 39 41) Our findings indicate that, although troublesome cross-reactions between individual strains belonging to different groups of betahaemolytic streptococci may occur it is possible to select strains for immunization and absorption that can be used for typing purposes as there is a restricted cross-reactivity between different Lancefield groups of these strains. Since the sensitivity of immunofluorescence tests differs from that of different precipitin tests, as antibodies not detected by precipitation are included, the strains to be used for immunization cannot be chosen on the basis of reports in the literature. We have shown, however that selected strains used for reference purposes are adequate for immunization and use in immunofluorescence A review of previously published papers (11 20 21 31–34 37 39 41–46) shows that others, using more or less pure FITC-labelled IgG preparations, have obtained highly conflicting results by comparing immunofluorescent detection of single groups of betahaemolytic streptococci with Lancefield's technique. It is generally agreed, however that group B is best studied by means of a pool of type-specific conjugates (31 33 44 46) Group D was not included in this study On the other hand, it has been thoroughly studied in immunofluorescence and is best identified if a pool of type-specific conjugates is used (38) To our knowledge, a well-defined immunofluorescence test of several groups, that compares favourably with Lancefield's technique, has not been published.

Several factors favour the use of our immunofluorescence technique for the rapid identification of groups A, B, C, E, F and G streptococci. Immunofluorescence shows a good covariation with Fuller's extract precipitin test. Many laboratories use immunofluorescence (11 49) or non-serological tests such as bacitracin sensitivity (13 14 49) and tripple test (48) for the identification of group A streptococci. According to the current evidence of pathogenicity of streptococci, especially of groups B, C and G (1 4 15, 26, 49) a simple and time saving method such as the one described here should be of value as it includes groups other than A in the list of rapidly determined streptococcal groups.

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REFERENCES

1 *Braunstein H Tucker E. B. & Gibson C. B.* Identification and significance of *Streptococcus agalactiae* (Lancefield Group B) Am. J Clin. Pathol. *51* 207–213 1969

Cherry W B. & Moody M D Fluorescent-antibody techniques in diagnostic bacteriology Bacteriol. Rev *29* 222–250, 1965.

3 *Cherry W B & Reimer C. R.* Diagnostic Immunofluorescence. Bull. Wld. Hlth. Org *48* 737–746, 1973

4 *Christensen K. K., Christensen P Flamholc, L. & Ripa, T* Frequence of streptococci of group A, B, C, D and G in urethra and cervix swab specimens from patients with suspected gonococcal infection Acta path. microbiol. scand. Sect. B, *82* 470–474 1974

5 *Christensen P., Kahlmeter G Jonsson S. & Kronvall, G* New method for the serological grouping of Streptococci with specific antibodies adsorbed to protein A-containing Staphylococci. Infect. Immunity *7* 881–885, 1973

6 *Christensen P & Kronvall, G.*: Capacity of

Group A, B, C, D and G Streptococci to agglutinate sensitized sheep red cells. Acta path. microbiol. scand. Sect. B 82 19–24 1974

7 *Curtis S. N. & Krause R. M.* Antigenic relationships between Groups B and G Streptococci. J. Exp. Med. *120* 629–637 1964

Dajani S. S. Rapid identification of beta hemolytic streptococci by counterimmunoelectrophoresis. J. Immunol. *110* 1702–1705 1973

Danielsson D.: The demonstration of *N. gonorrhoeae* with the aid of fluorescent antibodies. 5. A comparison of different techniques—absorption, one-step inhibition, and counter staining—for elimination of cross reactions. Acta Dermato-Venerol. *45* 61–73 1965

Danielsson D. & Forsum U. Diagnosis of Neisseria infections by defined immunofluorescence: methodological aspects and applications. Ann. N.Y. Acad. Sci. 1974. In press.

Edarer G. M. & Chapman S. Simplified fluorescent-antibody staining method for primary plate isolates of Group A Streptococci. Appl. Microbiol. *24* 160–161 1972.

12 *Edwards E. A. & Larson G. L.:* Serological grouping of hemolytic Streptococci by counterimmunoelectrophoresis. Appl. Microbiol. 26 899–903 1973.

13 *Estela L. A. & Shuey H. E.* Comparison of fluorescent antibody, precipitin, and bacitracin disk methods in the identification of Group A Streptococci. Am. J. Clin. Pathol. *40* 591–597 1963.

14 *Facklam R. R., Padula, J. F., Thacker L. G., Wortham E. C. & Sconyers, B. J.* Presumptive identification of Group A, B, and D Streptococci. Appl. Microbiol. *27* 107–113 1974

15 *Feingold D. S., Stagg N. L. & Kunz L. J.* Extrarespiratory streptococcal infections. N. Engl. J. Med. *279* 356–361 1968.

16. *Forsgren, A. & Forsum U.* Role of protein A in nonspecific immunofluorescence of *Staphylococcus aureus*. Infect. Immunity 2 387–391 1970.

17 *Forsum U.* Protein A from *Staphylococcus aureus* and its relation to bacterial serology. Acta Univ. Upsaliensis, 1972, p. 12.

18 *Forsum U.* Characterization of FITC-labelled F(ab') fragments of IgG and a rapid technique for the separation of optimally labelled fragments. J. Immunol. Methods 2 183–195 1972

19 *Forsum U. & Holmberg K.* Identification of *Streptococcus sanguis* by defined immunofluorescence. Caries Res. *8* 105–112, 1974

20 *Franek J. & Kubin V.* The use of fluorescent antibodies in the identification of streptococci. I. Identification of *Streptococcus pyogenes* group A. J. Hyg. Epidem. Microb. Immunol. *12* 82–90 1968.

21 *Franek J., Kubin V. & Prochazka, O.* The use of immunofluorescence in grouping of streptococci and in diagnosis of tularemia. I. Immunofluorescence studies of microorganisms. A. Application in bacteriology. Ann. N.Y. Acad. Sci. *177* 12–22, 1971

22. *Fuller A. T.:* The formamide method for the extraction of polysaccharides from haemolytic streptococci. Brit. J. Exp. Pathol. *19* 130–139 1938.

23 *Gaarfe H. & Wallin J.:* Studies in venereal disease. II. Improved diagnosis of gonorrhoea by the parallel use of conventional and L-phase media for culture. Br. J. Vener. Dis. *49* 505–507 1973.

24 *Goldman M.:* Fluorescent antibody methods. Academic Press, New York, 1968, p. 183–190.

25 *Holmberg, K. & Forsum, U.* Identification of Actinomyces, Arachnia, Bacterionema, Rothia, and Propionibacterium species by defined immunofluorescence. Appl. Microbiol. *25* 834–843 1973.

26 *Horstmeier C. & Washington J. A.:* Microbiological study of streptococcal bacteremia. Appl. Microbiol. 26 589–591 1973.

27 *Jones W. L. & Foster J. W.* Papain-treated globulins of specific and cross-reacting immunofluorescent staining. J. Bacteriol. *91* 984–986, 1966.

28. *Karakawa W. W., Krause R. M. & Borman E. K.:* Immunochemical aspects of the cross-reactivity between Groups A and G Streptococci as detected by the fluorescent antibody technique. J. Immunol. 94 282–288, 1965

29 *Krause R. M., Kindt T. J., Bokisch V. A. & Greenblatt J. J.* The antibody diversity following immunization with hemolytic streptococci. In: Haverkorn, M. J. (Ed.): Streptococcal disease and the community. Excerpta Medica, Amsterdam, 1974 p. 102–115.

30 *Kronvall, G.:* A surface component in group A, C, and G Streptococci with non-immune reactivity for immunoglobulin G. J. Immunol. *111* 1401–1406 1973

31. *Kubin V., Franek, J. & Prochazka O.:* Use of fluorescent antibodies for the identification of Streptococci. II. Determination of *Streptococcus agalactiae* (Group B). J. Hyg. Epidemiol. Microbiol. Immunol. *12* 315–323 1968

32. *Kubin V. & Franek J.* Annotation. Participation of type antigen R in the immunofluorescence reaction of Group B Streptococci. J. Hyg. Epidemiol. Microbiol. Immunol. *13:* 521–524 1969.

33. *Kubin V., Jelinkova J. & Franek J.:* The use of fluorescent antibodies in the identification of streptococci. III. Streptococcus B group

typing by means of the precipitation and immunofluorescence methods. J Hyg. Epidemiol. Microbiol. Immunol. *13* 382–388, 1969.

34 *Kuhn I & Franek J*. The use of fluorescent antibodies in the identification of streptococci. IV Determination of Group C Streptococci. J Hyg Epidemiol. Microbiol. Immunol. *15* 26–36, 1971

35 *Lancefield R. C*. The antigenic complex of *Streptococcus haemolyticus*. I Demonstration of a type-specific substance in extracts of *Streptococcus haemolyticus* J Exp. Med. *47* 91–103 1928.

36. *Lind I*. Identification of *Neisseria gonorrhoeae* by means of fluorescent antibody technique. Acta path. microbiol. scand. *70* 613–629 1967

37 *Moody M D., Ellis E C & Updyke E L*. Staining bacterial smears with fluorescent antibody IV Grouping streptococci with fluorescent antibody J Bacteriol. *75* 553–560 1958.

38. *Pavlova, M T Beauvais E., Brezenski F T & Litsky W* Fluorescent antibody techniques for the identifications of Group D Streptococci. Direct staining method. Appl. Microbiol *23* 571–577 1972

39 *Romero R. & Wilkinson. W*. Identification of Group B Streptococci by immunofluorescence staining. Appl. Microbiol. *28* 199–204 1974

40. *Rosendal, K* Grouping of hemolytic streptococci belonging to groups A, C and G A comparison between the results obtained by precipitation and by slide agglutination. Acta path. microbiol. scand. *39* 127–129, 1956

41 *Schimmelpfennig, H*. Zur Anwendung der Fluoreszenzserologie in der bakteriologischen Diagnostik. 2. Mitteilung: Nachweis und Differenzierung von Galtstreptokokken (*Strept agalactiae*) mittels fluoreszierender Antikörper Zentralbl. Veterinaermed. *11*: 407–417 1964

42 *Schueler R. L. Morehouse L. E. & Olson, L. D* A direct fluorescent antibody test for identification of Group E Streptococci Can. J Comp. Med. *37* 327–329 1973

43 *Smith T*. Clinical application of immunofluorescence I. Grouping β-hemolytic streptococci. J Bacteriol. *89* 198–204 1965

44 *Smith T*. Clinical application of immunofluorescence. III. Identification of Lancefield Group B Streptococci. Infect. Immunity 4 189–193 1971

45. *Smith T B & Wegner U*. Über die Gruppenbestimmung der β-hämolytischen Streptokokken. Arch. Hyg. *6* 625–630, 1971

46 *Tuomi, J & Narmi E*. Identification of *Streptococcus agalactiae* by the fluorescent antibody Acta vet. scand. *5* 50–55 1964

47 *Wadström T Nord C-E., Lindberg, A. L & Möllby R*. Rapid grouping of streptococci by immunoelectrophoresis. Med. Microbiol. Immunol. *159* 191–200 1974

48. *Wallerström, A* A simple biochemical "triple test" for preliminary identification of Group A Streptococci. Acta path. microbiol. scand. *56* 459–464 1962.

49. *Wannamaker L. W & Matsen J M* Streptococci and streptococcal diseases. Academic Press, New York, 1974

Acta path. microbiol. scand. Sect. B, 83 153–156, 1975

A METHOD FOR PRODUCTION OF ANTISERA TO HEPATITIS B ANTIGEN SUBTYPES, AND THE DISTRIBUTION OF SUBTYPES D AND Y AMONG NORWEGIAN HEPATITIS B PATIENTS

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Traavik, T A method for production of antisera to hepatitis B antigen subtypes, and the distribution of subtypes D and Y among Norwegian hepatitis B patients. Acta path. microbiol. scand. Sect. B, 83 153–156 1975

A method for production of antisera to hepatitis B antigen (HB_sAg) subtypes D and Y is described. The method is based on the coupling of HB_sAg to a colloidal silica gel (Aerosil) and the selective adsorption of unwanted antibodies from antisera with this preparation. Some results obtained with Norwegian hepatitis patient sera are included.

Key words. Hepatitis B antigen subtypes antisera subtype distribution.

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During the recent years, *LeBouvier* (6) d several other workers (2, 5 14) have vestigated the implications of the various bspecificities of hepatitis B antigen HB$_s$Ag) Special interest has been attached the determinants *d* and *y* The fact that ey were demonstrated to be mutually ex usive and virus strain specific (7 8) seemed make them valuable as sero-epidemiologi- l markers for virus B hepatitis. The produc on of reliable specific antisera to the sub- pe determinants has represented an annoy ng problem in this connection. The methods mployed for this purpose have been based n the adsorption of human or hyperimmune nimal antisera with heterologous HB_sAg n solution. Animals have been immunized with HB_sAg purified by various technique (3 5 6 14)

Stephan & Roka (referred to in 12) demonstrated the selective adsorption of serum lipoproteins to a colloidal silica gel. *Stephan* (12) also showed that the adsorption combined with UV light and beta-propiolactone treatment freed human sera from hepatitis hazards (12)

Siebke et al (10) demonstrated that HBAg in human sera is adsorbed to the colloidal silica gel "Aerosil" and presented some results concerning the optimal conditions under which such adsorption may take place

The present paper describes a simple method for the production of specific HB_sAg subtype human and guinea pig antisera. The procedure is based on the ability of "Aerosil"

to adsorb HB_sAg particles. Antisera produced have been used to subtype Norwegian hepatitis patient sera. Some results of this investigation are also presented.

MATERIAL AND METHODS

The procedure for HB_sAg subtype specific antiserum production is summarized in Fig 1

Immunization of guinea pigs with HB Ag subtypes D and Y (1)

↓

Antisera anti ad and anti ay

↓

Aerosil adsorption of sera to remove lipoproteins (3)

↓

Adsorption of sera with Aerosil HB_sAg to remove anti a antibodies (4) ← *Coupling of HB_sAg to Aerosil (2)*

↓

Specific anti d and anti y antisera.

Fig 1 Schematic presentation of the procedure used for HB_sAg subtype specific antiserum production. Figures in parantheses refer to the numbered periods in the material and methods section.

1 Immunization of Guinea Pigs

By means of reference reagents, kindly supplied by dr G. L. LeBouvier to be used in a modified Ouchterlony double diffusion test (ODI) (13) two patient sera were chosen for immunization purposes The patient H.K. was found to have a subtype Y (ay+ d–) HB_sAg-aemia, while the serum of S.A contained the D (ad+ y–) subtype.

The HBAg particles of these sera were pelleted by spinning 5 ml specimens at 100000 *g* for 1 h in a Christ ultracentrifuge with rotor SV-40 The pellet was washed once in 5 ml PBS pH 7 4 and centrifuged for another hour at 100000 *g* Finally the pellet was resuspended in 0.3 ml PBS and mixed thoroughly with an equal amount of Freund's adjuvant (Difco Lab Detroit 1 Michigan)

Young guinea pigs (300–500 g) were primarily immunized by an intra-dermal inoculation of 0.2 ml HB Ag-adjuvant mixture at three different sites on their backs. Two weeks later an identical booster dose was administered i.d. and a small serum sample was drawn for the demonstration of HBAb-activity by a modified Ouchterlony technique (13) These procedures were followed by weekly i.d. injections for 4 weeks. Two weeks after the last injection, the guinea pigs were bled by cardiac puncture. The antisera were tested by gel diffusion and the commercial Hepanosticon immuno-electro-osmophoresis set (abbr. IEOP) (Spectra Biologicals, Oxnard, Calif. 93030)

2 Coupling of HB_sAg to Silica Gel

Aerosil 380 (Degussa, Frankfurt a. Main) is, according to the manufacturer a colloidal silica composed of aggregated 7 nm primary particles with a surface area of about 380 m² per gram.

HB_sAg subtype Y serum (H.K.) and subtype D serum (S.A.) were mixed with 20 mg Aerosil per ml. In order to obtain efficient mixing, the tube contents were treated with a swirler (Cenco Instrumenten, MIJ NV., Breda the Netherlands) before being incubated for 16 hours (overnight) in a waterbath during automatical shaking (type 3047, J Köttermann, K. G Hanigsen, Germany) at 45 C. The tubes were placed horizontally in the water bath to keep the gel in suspension during the entire incubation period

The following morning, the gels were centrifuged for 30 min at 2500 g and washed 3 times in PBS to get rid of free antigen.

3 Removal of Serum Lipoproteins from Antisera

In order to avoid that free lipoproteins might compete with HB_sAg for Aerosil adsorption, the antisera (irrespective of species origin) were treated with Aerosil in the same way as HB_sAg sera. Earlier experiments (10) had shown Aerosil to reduce specific antibody activity to only a very slight degree.

4 Removal of Anti-a Determinant Antibodies from Antisera

Since all HB_sAg particles share a common determinant *a*, antibodies to this specificity must be removed to render antisera specific with regard to D and Y subtyping

In this final adsorption step Aerosil-linked Y HB_sAg was mixed with anti-*d* serum, while anti Y serum was mixed with Aerosil-linked *d* HB_sAg. It was found satisfactory to employ 20 mg Aerosil HB_sAg per ml antiserum. The swirler was utilized to ensure efficient mixing and the adsorption of anti-*a* antibodies took place in the water bath during vigorous shaking for 3 hours at 37° C. At the end of the incubation period, the mixtures were centrifuged for 30 min at 500 *g* and the antisera were pipetted off and kept at –20° C.

5 Removal of Antibodies to Human Serum Components from Antisera

Anti-human antibodies can be avoided if highly purified HBAg preparations are used for the immunization of guinea pigs. In this case a rather

de HBAg containing pellet was used and ac dingly some antibodies to human serum com- nents may have been provoked. If IEOP is to utilized for subtyping purposes, this does not resent a problem since the human serum glo- lins and the guinea pig antibodies will move the same direction during electrophoresis If her techniques were to be applied, however (gel ecipitation, complement fixation, passive haem- glutination, radio immuno assay) the presence anti-human antibodies must be avoided. This was hieved by the following procedure: Guinea pig HB_sAg subtype antiserum and a known HB_sAg/ b negative human serum (tested by radio im- muno assay) were mixed in equal parts and in- bated at 37 C for 1 h. The serum mixture was en diluted ½ in PBS and layered on a pre- ormed 10–40 per cent sucrose gradient and cen- ifuged for 17–18 hours at 26000 rev/min in ernaeus Christ Vacufuge with rotor 840/135TL The gradient was unloaded in fractions of 10 drops rom the bottom The fractions were analysed for nti-human antibodies by a modified Ouchterlony echnique (13) using a known HB_sAg/Ab nega- tive human serum as antigen. The fractions con- taining HBAb demonstrated no activity directed against human serum components

6 Patient Sera for HB_sAg Subtyping

Sera from 106 patients with HB_sAg-aemia diag- nosed by IEOP and CHI during the period 1972– 74 were randomly chosen N anamnestic and/or clinical information was available to the in estiga- tors until after the subtypings had been performed. From 31 of the patients, 2 or more sera drawn at different times were available. The total number sera subjected to subtyping by IEOP and CHI is 161

RESULTS AND DISCUSSION

he guinea pig HB_sAg subtype antisera ob- ined by the procedure described are con- dered specific and usable in subtyping due the following facts

1 The sera have proved to give clear-cut egative reactions when tested against he erologous HB_sAg preparations by IEOP and CHI both Le BOUVIERS references and atient sera.

2 The precipitating antibodies are re moved by adsorption with homologous anti gen preparations.

3. In CHI lines of identify with the refe rence sera from dr C L. *LeBouvier* are pro- duced.

4 As regards 31 HB_sAg positive patients, more than one serum was available 2 sera were available from 14 patients 3 sera from 10 patients 4 sera from 5 patients 5 sera from 1 patient 6 sera from 1 patient. In every case, all the sera from one patient have demonstrated the very same subtype determi nant.

Among the total of 106 patients, 42 de monstrated HB_sAg subtype D and 51 were found to have subtype Y (Table 1) The mean age of patients presenting D subtype was 39 years, ranging from 75 to 16 years. The mean age of patients with Y subtype was 20 years, ranging from 14 to 28 years.

TABLE 1 *Distribution of HB_sAg Subtypes among Norwegian Antigen Positive Patients Chosen at Random*

Total number	Subtype D (ad+ y–)	Subtype Y (ay+ d–)	Untypeable
106	42	51	13

Some data applying to the 2 groups of pa tients are summarized in Tables 2 and 3. The distribution of subtypes in this series proved to be very similar to that reported from other European countries (4 9 11 14) and from North America (3 8, 15) Among drug abusers, the incidence of HB_sAg subtype Y (ay+ d–) was found to be very high, while the D subtype (ad+ y–) predominated among sporadic cases of hepatitis.

A human antiserum against HB_sAg (A.O) giving precipitation lines in IEOP in a dilu- tion of 1/32, was concentrated 10 times by Lyphogel (Gelman) (1) and subjected to the Aerosil adsorption procedure. This serum demonstrated antibodies to subtype D HB_sAg in a dilution of 1/4 by IEOP and showed identity to the guinea pig anti D serum and LeBouvier s reference serum by gel precipita tion (13)

In conclusion, the procedure described in this report offers a specific and reliable method for the production of HB_sAg subtype antisera from human or animal sources. It

TABLE 2. *Patients with HB_sAg Subtype D (ad + y–) Diagnostic/Anamnestic Information Obtained from the Attending Physicians*

	Number	Per cent
Sporadic cases.		
Exposition not known.	26	62
Inoculations? (Therapeut. inj./hospital accidents)	5	12
Hepatitis epidemics?	4	10
No informations, HBAg test required	4	10
Visits to the Mediterranean countries	1	2
Voluntary blood donor Susp. virus transmission	1	2
Diarrhoea	1	2
Total	42	100

TABLE 3. *Patients with HB_sAg Subtype Y (ay + d–) Diagnostic/Anamnestic Information Obtained from the Attending Physicians*

	Number	Per cent
Intravenous drug abuse	43	84
Suspected drug abuse	5	10
Visits to the Mediterranean* countries	2	4
Sexual contact with drug abuser	1	2
Total	51	100

* Including one haemophiliac frequently receiving cryoprecipitates.

can be carried out by means of relatively simple equipment. Provided that HB_sAg of known subspecificities are available, the method might also prove valuable in the production of antisera directed against other HBAg determinants.

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REFERENCES

1 *Aithawal, R. S & Peters R L.* Hepatitis-associated antigen Improved sensitivity in detection. Amer J Clin. Pathol. 55 262–268 1971

2. *Bancroft W H Mundon, F K. & Russel, P K* Detection of additional determinants of hepatitis B antigen. J Immun. 109 842–848, 1972.

3 *Gordin I., Berbersen, M Stevenson, D & Redeker A G.* Distribution of hepatitis B antigenic determinants in different forms of viral hepatitis. J Infect. Dis. 126 569–573, 1972

4. *Iwarson S Magnius L., Lindholm A & Lundin P* Subtypes of hepatitis B antigen in blood donors and posttransfusion hepatitis Clinical and epidemiological aspects. Brit. Med. J 1 84–87 1973.

5. *Kim C Y & Tilles J G.* Immunologic and electrophoretic heterogeneity of hepatitis-associated antigen. J Infect. Dis. 123 618–628, 1971

6. *LeBouvier G L.* The heterogeneity of Australia antigen. J Infect. Dis. 123 671–675, 1971

7 *LeBouvier G L. McCollum R. W Hierholzer W J., Irwin G R., Krugman S & Giles, J P* Subtypes of Australia antigen and hepatitis-B virus. JAMA 222 928–930, 1972.

8. *Mosley J W., Edwards V W Meihaus, J E. & Redeker A. G* Subdeterminants d and y of hepatitis B antigen as epidemiological markers. Amer J Epidem. 95 529–535, 1972.

9 *Nielsen, J O., LeBouvier G L. & the Copenhagen hepatitis acute program* Subtypes of Australia antigen among patients and healthy carriers in Copenhagen N Engl. J Med. 288 1257–1261 1973

10 *Siebke J C Kjeldsberg E. & Traavik, T* On the adsorption of Australia antigen to colloidal silica, and some possible applications. Acta path. microbiol. scand. Sect. B, 80 935–936 1972.

11 *Skinhøj P* Hepatitis-associated antigen, subtypes D and Y an epidemiological and clinical evaluation. Scand. J Infect. Dis. 5 85–89 1973

12 *Stephan W* Hepatitis-free and stable human serum for intravenous therapy Vox Sang. 20 442–457 1971

13 *Traavik, T., Siebke J C & Kjeldsberg E.* A sensitive modification of the Ouchterlony technique. Detection of hepatitis associated antigen in a closed hexagonal system. Acta path. microbiol. scand. Sect. B 80 773–774, 1972.

14 *van Kotten Doorschodt H J van der Akker, R & Gispen R.* Determination and distribution of two types of hepatitis associated antigen. J Infect. Dis. 126 117–122, 1972.

15 *Wenzel P R LeBouvier G. L. & Beam, W E.* Drug abuse and viral hepatitis in marines. JAMA 220 707–709 1972

BRIEF REPORT

SEPARATION OF PATHOGENIC *TREPONEMA PALLIDUM* FROM HOST RABBIT TISSUE TO OBTAIN VIRULENT AND VIABLE MICROORGANISMS

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Rathlev T Separation of pathogenic T eponema pallidum from host rabbit tissue to obtain virulent and iable microorganisms. Acta path. microbiol. scand. Sect. B 83 157–160 1975

T pallida extracted from infected rabbit testes were purified from different tissue particles by equilibrium density centrifugation at 25 C after layering on a column of Ficoll-sodium metrizoate solution. After centrifugation, 3? per cent of the treponemes were present in a visible band and about 57 per cent were concentrated in the interface between the Ficoll-Na metrizoate cushion and treponeme-containing medium. The treponemes were viable irulent and retained their morphological integrity infectivity and antigenicity Purity of the different fractions was studied by immunodiffusion experiments. The purified preparations of virulent, viable treponemes will provide a better antigen in the FTA-ABS tests and possibly in the TPI and TPHA tests in cellular immunity studies, electron microscopic investigations of the ultrastructure of treponemes, vaccination studies and in tissue culture investigations

Key ords Pathogenic T ponema pallidum separation rabbit tissue

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It is well known that virulent T eponema pallidum cannot be cultivated in itro as yet. Rabbits are commonly used to propagate the organisms in ivo and to obtain treponemal antigen for serological tests and a ariety of experimental studies.

The suspension of T pallidum extracted from the infected tissue contains erythrocytes, leucocytes, permatozoa, particulate tissue and should be freed rom these particles as much as possible when the organisms are to be used as antigen in the TPI FTA ABS and the TPHA tests. Removal of the host tissue is essential when the treponemes are to be used in accination experiments, in tissue culture studies and as an antigen or target cells to investigate cellular immunity mechanisms in treponemal diseases

Different purification procedures used so far include series of differential centrifugations (3) density gradient centrifugation on potassium tartrate (4) continuous particle electrophoresis (5) and filtration in millipore chambers (1) These procedures were successful to various degrees as far as removal of host tissue was concerned, but had little success in obtaining viable and virulent organisms.

The procedure described here consists of centrifugation for only 25 minutes to yield motile and irulent treponemes purified from the host tissue. The organisms thus obtained were li e and virulent and retained their morphological integrity and infectivity

Material and Method

T pallid m (Nichol's strain) was cultivated in adult, white male rabbits by intratesticular injection. After approximately ten days, when orchitis had developed, the animal were exsanguinated,

and the testes were removed under aseptic conditions. Each testicle was cut across its length without dividing it into two pieces, placed flat on the opened side in a sterile petri dish, and cut crosswise at 2–3 places to produce a "butterfly" shaped tissue. The latter was washed with about 10 ml sterile saline to remove large bulk of the lipid and soft tissue particles. Most of the treponemes, however still remained in the fibrous tissue which was shaken at 34 C for 30 minutes in a petri dish containing 15 ml of the medium prepared as follows

Powder McCoy's 5a, modified medium (Grand Island Biological Company) was reconstituted in 25 mM HEPES buffer (calbiochem, Cat. No. 391338) Phenol red was present as pH indicator and heat-inactivated fetal calf serum was added to a final concentration of 15 per cent (v/v)

The average number of treponemes per field was determined after examining ten fields in a calibrated darkfield microscope and the number of organisms per milliliter of the suspension was calculated by using an exact volume (5 microliters) of the suspension and taking into account the area of the cover slip and the diameter of the field The number of treponemes in the final suspension was adjusted to 1×10^7 organisms per ml either by addition of more med um or by extracting more treponemes from the infected tissues.

Ficoll-sodium metrizoate solution Eighteen gms of Ficoll mol. wt. 400 000 (Pharmacia, Sweden) were dissolved in sterile, distilled water by stirring on a magnetic stirrer at room temperature and the volume was adjusted to 200 ml Sodium metrizoate ("Triosil" from Glaxo Lab Ltd., Greenford, England) was adjusted to a concentration of 33.9 per cent in sterile distilled water and mixed with the Ficoll solution in the ratio of 5 12 (/v) Other derivatives of this radiocontrast chemical such as sodium diatrizoate can also be used. Sufficient volume of the Ficoll-sodium metrizoate solution was diluted by mixing 4 parts with approximately 1 part olume of sterile distilled water so that the density of the final solution was 1 069 ± 0.001 and the refractive index was 1 3530 These determinations were done by using calibrated micro pipettes. The final solution was sterilized by filtration through Nalgene filter unit, 0 2 micron, plain membrane, and was stored at 4 C in dark. The temperature of the solution was brought up to 25 C just before use.

Suspension of the treponemes in McCoy's medium was centrif ged for 5 minutes t room temperature at approximat ly 500 × g to sediment erythrocytes and leucocytes and the supernatant was gently removed.

Purification was carried out in a Beckman centrifuge model L-2-65B, with SW 27 swing bucket rotor using the 1 59 cm × 10 16 cm set of buckets with a volume capacity of 17 ml per tube and a total capacity of 102 ml with six tubes.

Five milliliters of Ficoll-Na metrizoate solution at 25 C was added to each centrifuge tube and ten ml of *T pallidum* suspension in McCoy's HEPES medium with 15 per cent fetal calf serum, was layered very gently on top of the solution without disturbing the interface. The latter was clearly visible as a sharp line between the reddish medium on top and the colourless solution at the bottom of the tube. It was important to maintain the temperature of the solutions in the tubes between 25–34 C throughout the operation. The tubes were placed in the buckets which were kept at 25 C for about an hour prior to the centrifugation, which was carried out at 4000 rev/min (2000 × g at R_{av} of 11.57 cm) for 20 minutes at 25 C.

At the end of the centrifugation, the tubes were gently removed and placed in a 34 C incubator. A white band containing 1/3 of all treponemes was clearly isible just above the interface. The white layer (0.5 ml) was removed very gently with a Pasteur pipette without disturbing the interface or picking up the Ficoll-Na metrizoate cushion. This procedure was facilitated by bending in a flame approximately 5 mm of the narrow end of the Pasteur pipette at right angle to its length, so the removal of the heavy cushion was avoided Twenty 0.5 ml fractions—starting from the interface upwards—were collected and were kept in 34 C incubator The first two fractions were immediately diluted by adding an equal volume of the McCoy's-HEPES medium to dilute the small amount of the heavy cushion that might have been picked up and also to dil te the concentrated bulk of *T pallid m* in these two layers. Each fraction was examined by darkfield microscopy and the number of treponemes in each 0.5 ml fraction was calculated by counting the number per field.

Immunodiffusion experiments (2) were carried out to show the absence or presence of tissue material in the fractions that appeared purified on the basis of microscopic examination. Sonicated and solubilized particulate material from these fractions was used as the antigen and serum from an immunized goat as the antiserum. The goat was injected once month for a period of one year with sonicated, normal rabbit testis tissue which was freed from blood cells and spermatozoa by centrifugation, and from rabbit serum by washing with buffered saline.

The infectiv ty of the treponemes from the first six fractions which did not show presence of tissue was studied by intratesticular injection of three rabbits. The number of treponemes was adjusted to 1 × 10 per ml and each testicle was injected with 1 ml of the suspension. As controls, three normal rabbits were injected intratesticularly with (a) McCoy's-HEPES-fetal calf serum and (b)

TABLE 1 *Results Obtained in a Typical Purification Experiment*

Fraction No. (0.5 ml)	No. of treponemes per fraction	Per cent motile	Fine tissue (No. of precipitin lines)
1	22 × 10⁶	95–98	none
2	10 × 10⁶	100	none
3	5 × 10⁶	100	none
4	5 × 10⁶	100	none
5	12 × 10⁶	100	none
6	3 × 10⁶	100	none
7	4 × 10⁶	100	2
8	2.5 × 10⁶	100	2
9	1 × 10⁶	100	3
10	1 × 10⁶	100	3

three normal rabbits with the above medium containing 5 per cent (v/v) Ficoll-Na metrizoate solution. The volume injected per testis was 1 ml in all cases.

The antigenicity of the purified treponemes was tested in the FTA-ABS (6) test. Fractions 1 to 6—from the interface upwards—were pooled, three parts by volume of phosphate buffered saline (pH 7.2) was added and the treponemes were sedimented in a Sorvall centrifuge at 15,000 rev/min for 30 min at 25 C. The sedimented organisms were resuspended in the special buffer used for this test (6) to yield a suspension with four to six treponemes per field for fluorescent microscopy.

Results and Discussion

After equilibrium density centrifugation and throughout the period involved in counting the treponemes, injecting them intratesticularly in rabbits and in the preparation of the antigen for the serological *test*, *T. pallidum* remained actively motile and retained morphological integrity. Important reasons for this result are probably the rapidity of purification, avoidance of several centrifugations, maintenance of the temperature between 33–34 C and the medium used for the extraction and suspension of the treponemes.

A typical purification experiment where 1 × 10 treponemes per ml of suspension were layered in one tube, gave the results described in Table 1.

These results show that approximately 57 per cent of the treponemes from the original suspension were present in the first six fractions or three ml suspension above the interface. The white band with treponemes contained about 32 per cent of the total number of organisms layered in the tube.

Immunodiffusion experiments *showed presence* of lighter fine particulate tissue mostly in the fractions at the top of the tube. Erythrocytes, leucocytes, and spermatozoa if at all present after the usual low speed centrifugation, sedimented as a button at the bottom of the tube covered with Ficoll-Na metrizoate solution.

The older procedures of density centrifugations were designed to collect almost all of the organisms in one layer and exclude all other biological material. This was achieved with the result of losing the lability and virulence of the organisms. Now emphasis should be placed on obtaining biologically active and virulent organisms, separated from the other tissue components even though their recovery may not be 100 per cent. This approach takes into account that microorganisms, cells or membranous organelles possess variable sizes and densities and their buoyancy is influenced by the physical-chemical properties and osmotic activity of the solutions they traverse. Therefore, it is reasonable to expect and obtain different layers of motile treponemes with and without contaminating tissue depending on its buoyant equilibrium density.

Besides the conditions of the centrifugation, use of the special longer set of buckets—1.59 cm diameter and the longer path length (10.16 cm) of the tubes facilitated separation of *T. pallidum* from the heavier cells and the lighter tissue particles.

The treponemes from all the fractions were virulent and produced orchitis in rabbits approximately 10 days after intratesticular injection of 1 × 10⁷ treponemes per testis. In rabbits, intratesticular injection with either the medium alone or with Ficoll Na metrizoate, did not produce any abnormal reaction. These rabbits when challenged two weeks later with virulent treponemes, produced orchitis as usual *after eight* to ten days. Thus, Ficoll or Na metrizoate or the components of the medium did not seem to produce any toxic or stimulatory reaction when injected in animals.

Immunodiffusion experiments with sonicated, *solubilized rabbit* testes tissue as antigen and goat anti-rabbit-testes-tissue serum, showed presence of precipitin components. However, with the sedimented and sonicated material from fractions 1 to 6, no precipitin lines were obtained. The amount

of the fine tissue seemed to increase gradually in the fractions in the top part of the centrifuge tubes.

In the FTA-ABS tests, purified treponemes from fractions 1 to 6 proved to be a much better antigen due to the absence of the contaminating debris which produces background fluorescence. Also their morphological integrity was in no way affected by centrifugation over a column of Ficoll-Na metrizoate.

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References 1 *Chandler F W & Clark, J W:* Appl. Microbiol. 19 326–328, 1970.—2. *Ouchterlony Ö.,* Acta path. microbiol. scand. 32 231–240 1953.—3 *Portnoy J & Magnuson, H J* Ame. J Clin. Path. 26 313–322, 1956.—4. *Reinlev T & Pfau C J.,* Scand. J Clin. Lab. Invest. 17 130–134 1965.—5 *Schmale J D Kellog, D S., Miller C E. Schammel, P & Thayer J D:* Appl. Microbiol 19 287–289 1970.—6. U.S. Dept. Hlth. Ed. Welf., Manual of Tests for Syphilis, 1969

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EXAMINATION OF FIMBRIATION OF SOME GRAM NEGATIVE RODS WITH AND WITHOUT TWITCHING AND GLIDING MOTILITY

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Henrichsen, J. & Blom, J. Examination of fimbriation of some gram-negative rods with and without twitching and gliding motility. Acta path. microbiol. scand. Sect. B 83: 161–170, 1975.

Negatively stained preparations of 30 different strains of gram-negative rods representing 20 different taxa were examined in the electron microscope. Thirteen of the strains studied exhibited twitching and six of the strains exhibited gliding motility. Additionally, non-twitching substrains of two of the twitching strains and a non-gliding substrain of one of the gliding strains were examined. A variety of cultural media, preparations for negative staining and negative stains were used. It was found that all strains with twitching motility possessed fimbriae, the diameter of which was approximately 50 Å in all but one strain; the fimbriae of this strain had a diameter of approximately 40 Å. The fimbriae were judged to be of polar origin in all cases where the origin could be determined with certainty. On none of the strains without twitching motility could fimbriae be demonstrated. Only one of the six strains with gliding motility possessed fimbriae.

Key words: Gram-negative rods; twitching motility; polar fimbriae.

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The type of bacterial surface translocation called twitching motility has been described previously (10, 16). As the mechanism of this type of motility is unknown, it is of considerable interest that a correlation between fimbriation and twitching motility has been demonstrated, first in the three species of *Moraxella* (13) and then in strains of *Acinetobacter calcoaceticus* (12). While the origin of the fimbriae of the *Moraxella* species was not precisely determined it was shown, by the aid of improved methods for preparation of specimens for negative staining, that the fimbriae of strains of *A. calcoaceticus* with twitching motility had a polar origin (12). Polar fimbriae have been observed previously on strains of *Pseudomonas aeruginosa* (1, 9, 23), *Ps. echinoides* (14) and *Caulobacter* sp. (20), and also on strains of six out of 15 different species of *Pseudomonas* examined by *Fuerst & Hayward* (9). On three anaerobic, corroding bacterial strains, polar fimbriae were demonstrated by *Jackson et al.* (15) and their presence was "associated with ability to form spreading colonies" but "although motility was strongly suggested" it was not detected.

With the purpose of verifying, or falsifying that possession of polar fimbriae is a necessary prerequisite for twitching motility in all cases, we have extended our electron microscope studies to comprise representatives of all species known at present to exhibit twitching motility (11)—if information with regard to their fimbriation was not already available in the literature. For comparative purposes we included in this study two non-twitching substrains of twitching wild-type strains as well as representatives of species of *Pseudomonas* known to be without twitching motility (11). Some non twitching strains of *Flavobacterium* sp. that spread by sliding (11) were included because *McMeekin et al.* (17) had observed twitching motility in some gram-negative, yellow-pigmented rods. One representative of such yellow pigmented rods with twitching motility was examined too. A strain of *Alcaligenes odorans* and one of *Moraxella phenylpyruvica* both with sliding motion, were also included. Finally we included a number of gliding strains to look for the occurrence of fimbriae on these organisms because the mechanism of gliding motility is likewise unknown (10). The results obtained are presented in this paper. Briefly we observed polar fimbriae on all strains that exhibited twitching motility.

Subsequently the opposite question was posed: Do all strains which are known to possess polar fimbriae also exhibit twitching motility? The elucidation of this forms part of a study that will be published separately (11).

MATERIALS AND METHODS

The Bacterial Strains studied are listed in Tables 1 and 2.

Agar Plate Microscopy was carried out as described previously (10) and was always performed before transferring the bacteria to specimen grids in order to check whether the bacteria selected for electron microscopy were exhibiting twitching or gliding motility or neither.

Media

Meat extract agar plates (MEA), 5 per cent blood agar plates and cytophaga agar no. 62 (also used in its liquid equivalent) were made as described before (10).

Cystine-haemin-nutrient agar plates (CHA) were made according to *Jackson et al.* (15).

Salt agar plates (SA) were made of 0.1 per cent meat extract (Lab-Lemco), 0.25 per cent peptone (Difco), 0.05 per cent yeast extract (Difco), 1.5 per cent sodium chloride, 1.0 per cent Bacto-agar (Difco) and 1 per cent Hutner's mineral base (4) with a final pH adjusted to 7.2. This medium was also used in its liquid equivalent.

Incubation. Culture plates were incubated in plastic bags or in closed jars with a layer of water in the bottom in order to ensure a humid atmosphere. Anaerobic strains were incubated in an anaerobic jar (Baird and Tatlock, Ltd). The temperature used was 30° C for most strains. However strains A 353, NCMB 263, 1-R 41, RL-4, NCMB 247, H 556 and U 67 were incubated at 22° C and strains 310 A, 333, H 138, 017 A and 316 were incubated at 35° C.

Electron Microscopy was performed as described previously using mainly the "drop method" and the "wash off method" for bacteria grown on solid media (12). Briefly the principle of these methods is to secure a high proportion of bacteria in the exponential growth phase on the grid in a concentration suited for electron microscopy: 1) by applying a drop of the negative stain either on the outer part of the spreading zone or anywhere on a plate covered by microcolonies, and placing the grid on top of this drop, and 2) by washing off the microcolonies of two to five plates with the negative stain and then place the grid on top of a drop of the resulting bacterial suspension. In some cases it was found advantageous to use ammonium acetate instead of the negative stain for the first step of both methods, suck off excessive amounts of suspension and thereafter negatively stain the bacteria remaining on the grids with ammonium molybdate or other heavy metal salts. Most often 1 per cent (w/v) ammonium molybdate adjusted to pH 7.4 with NH_4OH was used, but in some cases satisfactory preparations were not obtained by this stain; then 1 per cent (w/v) phosphotungstic acid (PTA) adjusted to pH 7.8 with KOH or 1 per cent (w/v) uranyl acetate (UAc) with a pH of 4.0 was tried.

Measurements of the diameters of fimbriae were made directly on single fimbriae on prints at a final magnification of 90,000 × as described before (12).

RESULTS

The results are summarized in Tables 1 and 2.

From Table 1 it is immediately evident that all strains with twitching motility possessed fimbriae and that non-twitching strains

TABLE 1 *List of Strains Studied and Correlation between Twitching Motility and Fimbriation (Strains with Gliding Motility are Listed in Table 2)*

Strains studied	Strain designation[a)]	Spreading growth[b)]	Twitching motility	Fimbriae[c)]
Alcaligenes odorans	H 1079	+	—	—
"Bacteroides corrodens"	316	+	+	+ +
Eikenella corrodens	310A SC[d)]	+	+	+ + **
Eikenella corrodens	310A-N[e)]	—	—	—
Eikenella corrodens	333-SC[d)]	+	+	+ *
Eikenella corrodens	333-N[e)]	—	—	—
Flavobacterium meningosepticum	ATCC 13253	—	—	—
Flavobacterium meningosepticum	1/H 86	—	—	—
Flavobacterium sp	8/J 207	+	—	—
Flavobacterium sp.	17/J 232	+	—	—
Flavobacterium sp.	A 280	—	—	(+)
Flavobacterium sp.	U 120	+	—	—
Moraxella phenylpyruvica	23633/69	+	—	—
Pseudomonas acidovorans	AB 1232	+	+	+ +
Pseudomonas aeruginosa	H 1025	+	+	+
Pseudomonas aeruginosa	H 1091	+	+	+ +
Pseudomonas aeruginosa	PJ 630	+	+	+
Pseudomonas cepacia	H 1020	—	—	—
Pseudomonas pseudoalcaligenes	AR 79	+	+	+
Pseudomonas putida	H 1021	—	—	—
Pseudomonas putrefaciens	AB 769	—	—	—
Pseudomonas putrefaciens	AB 1380	+	+	+ + *
Pseudomonas solanacearum	017 A	+	+	+ +
Pseudomonas stutzeri	H 1022	+	+	+ +
Phenon 3 of *Thornley* (21)	A 333	+	+	+ + *
Marine, yellow-pigmented rod	NCMB 263	+	+	+ +

a) ATCC, American Type Culture Collection, Rockville, Maryland USA. NCMB, National Collection of Marine Bacteria, Torry Research Station, Aberdeen, Scotland. Strains 310 A, 333 (= NCTC 10596) and 316 were isolated by *Eiken* (6 7). Strains 1/H 86 8/J 207 and 17/J 232 were isolated by *Olsen* (18). Strain 23633/69 was isolated by *B. Frederiksen* Department of Medical Bacteriology County Hospital of Aalborg South, Aalborg, Denmark. Strain 017 A was received from *A C Hayward* Department of Microbiology University of Queensland St. Lucia 4067 Australia. All other strains are from the collection of the Department of Diagnostic Bacteriology Statens Seruminstitut, Copenhagen, Denmark.

b) Gliding (10) is the cause of the spreading growth in those strains listed that do spread and do not exhibit twitching motility

c) + + = many fimbriae + = a few (+) = maybe a few but difficult to evaluate with certainty due to slime threads means that the origin of the fimbriae has not been decisively determined, but fimbriae appear to be concentrated near the poles and the regions of division. * means that the origin of the fimbriae is unknown. Absence of asterisk signifies that the fimbriae definitely have a polar origin.

d) Strain variant with spreading and corroding colonies.

e) Strain variant with non-spreading and non-corroding colonies.

were non-fimbriated. The diameters of the fimbriae encountered on cells of the different strains studied were approximately 50 Å with the exception of the anaerobic polarly flagellated strain 316, which carried fimbriae with a diameter of approximately 40 Å. In all but one strain (310A SC, *vide infra*) the fimbriae were judged to be of polar origin, although in some strains this was not decisively determined, but only assumed to be most likely because the fimbriae apparently were most plentiful near the poles and the regions of division of the cells.

Because the fimbriae undoubtedly are

easily detached from the cells we did not try to evaluate systematically the average number of fimbriae per cell but only made a rough estimate of whether the cells of a given strain had few or many fimbriae.

Fimbriae were never seen on all cells in a given preparation and quite often only on a minority of the cells. Before a strain was registered as non-fimbriated it was therefore usually examined on more than one occasion and always by means of optimal preparative methods, i.e. the "wash off method" the "drop method" or negative staining of young liquid cultures.

The sliding strains studied did not possess fimbriae.

The polar origin of the fimbriae of *Pseudomonas solanacearum* strain 017 A is clearly seen in Fig 1 which shows a cell from a preparation of a 20 hour-old liquid culture. Figs. 2 and 3 show the vast masses of fimbriae found in preparations of this strain made by the "drop method" applied to plates covered with microcolonies consisting of twitching cells only. Although not very clearly discernible the appearance of the fimbriae suggests the existence of substructures of a periodic nature, i.e. these fimbriae conceivably are composed of repeating subunits (Fig 2). When stained with uranyl acetate (pH 4) laterally adherent aggregates of fimbriae were predominant (Fig 3). Other examples of polarly fimbriated *Pseudomonas* strains are given in Figs 4 and 6 while Fig 5 shows an example of a non-fimbriated strain of *Ps cepacia*. In some of the preparations of the non-twitching *Ps putrefaciens* strain AB 769 we found a few probably peritrichously arranged, fimbriae with a diameter of approximately 30 Å, i.e. nearly half as thin as the polar fimbriae found on the twitching strain *Ps. putrefaciens* AB 1380.

None of the *Flavobacterium* strains examined possessed fimbriae (Figs. 7 and 8) but strain A 280 was impossible to evaluate with a reasonable degree of certainty due to the presence of heavy masses of slime around the cells (Fig 8).

Also the sliding strain, 23633/69 of *Moraxella phenylpyruvica* was found to be without fimbriae as shown in Fig. 9 which was obtained from a "suspension method" preparation. Specimens prepared by the "wash off method" gave identical results.

It was possible to split the two strains of *Eikenella corrodens* into a twitching (as well as spreading and corroding) and a non-twitching (non-spreading and non-corroding) variant which were shown to be fimbriated and non-fimbriated, respectively (Figs. 10 and 11). The cells of strain 310A variant SC were embedded in such heavy masses of detached fimbriae that it was impossible to trace the origin of the fimbriae from the cells (Fig 10). The same kind of substructure may be discerned in these fim-

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Unless otherwise indicated all figures show material negatively stained with 1 per cent ammonium molybdate. Incubation temperatures for individual strains are given under Materials and Methods. The bar on all micrographs represents 0.1 μm and all the figures are shown at a final magnification of 90 000 ×

Fig 1 Pseudomonas solanacearum strain 017 A. 20 hour-old liquid no. 62 culture Polar region of a cell with a tuft of polar fimbriae. Detached flagella are also seen.

Fig 2 Ps. solanacearum strain 017 A. "Drop method" preparation from 15 hour-old microcolonies on cytophaga agar no. 62. Detached fimbriae with a just discernible substructure.

Fig 3 Same as for Fig. 2, but negatively stained with uranyl acetate. Laterally adherent aggregates of fimbriae predominate

Fig 4 Ps. acidovorans strain AB 1232. 20 hour-old liquid no. 62 culture. Polar fimbriae and a flagellum are seen.

Fig 5 Ps cepacia strain *H 1020* "Wash off method" preparation from 18 hour-old microcolonies on cytophaga agar no. 62 Only flagella, but no fimbriae, are seen.

Fig 6. Ps. aeruginosa strain H 1091 "Drop method" preparation from 10 hour-old microcolonies on cytophaga agar no. 62. Fimbriae are seen radiating from the poles of two cells

Fig 7 Flavobacterium sp. strain 8/J 207 20 hour-old liquid no. 62 culture Blebs and "slime threads" but no fimbriae, are seen.

1

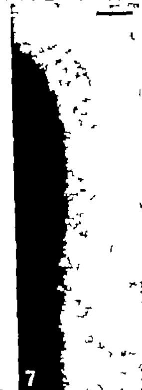
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TABLE 2. *List of Strains Possessing Glidi g Motility and Results of Search for Fimbriae*

Strains studied	Strain designationᵃ)	Spreading growth	Gliding motility	Fimbriaeᵇ)
Cytophaga columnaris	1-R 43	+	+	(+)
Cytophaga succinicans	RL-8	+	+	—
Cytophaga sp. (marine)	NCMB 247	+	+	—
Cyt ph ga sp.	H 356	+	+	—
Cytophaga sp.	U 67	+	+	—
I itreoscilla sp.	UWO 390	+	+	+ +**
I itreoscilla sp.	UWO 390-N	—	—	+**

ᵃ) Strains 1 R 43 and RL-8 were received from *E. J Ordal*, Department of Microbiology School of Medicine, Unh ersity of Washington, Seattle Washington, USA. Strain UWO 390 was received from *R. G E. Murray* Department of Bacteriology and Immunology University of Western Ontario, London, Canada the non-gliding variant UWO 390-N was isolated by one of the authors (J H.)

ᵇ) For meaning of symbols: see explanation given in Table 1

F g 8 *Flavobacterium* sp. strain A 280. "Drop method" preparation from 15 hour-old microcolonies on cytophaga agar no 62. Fimbriae are not seen with certainty but due to the network of "slime threads their presence cannot be completely excluded.

Fig 9 *Moraxella phenylpyruvica* strain 23633/69. "Suspension method" preparation from spreading zones of 48 hour-old colonies on 5 per cent blood agar plates. Many blebs, but no fimbriae are visible

F g 10 *Eikenella corrodens* strain 310A-SC. "Drop method" preparation from spreading zones of 96 hour-old colonies on CHA. Large bundles of laterally aggregated, long and straight fimbriae of undeterminable origin and a just discernible substructure are seen

Fig 11 *E. corrodens* strain 310A N Wash off method" preparation from 72 hour-old microcolonies on CHA. Some blebs, but no fimbriae are isible.

Fig 12 Strain 316_r. "Suspension method" preparation from spreading zones of 72 hour-old colonies on CHA. The cell has a tuft of polar fimbriae. A bundle of detached fimbriae and a piece of a flagellum are isible in the right hand part of the field

F g 13 Same as for Fig. 12. One polar flagellum and tw polar fimbriae re seen on this cell.

F g 14 Strain NCMB 263. 48 hour-old liquid SA ulture A tuft of polar fimbriae is seen on this cell.

F g 15. Strain NCMB 247 48 hour-old liquid SA culture negatively stained with PTA. Blebs and "slime threads but no fimbriae are isible.

brae as in those of *Ps solanacearum* shown in Fig. 2.

In strain A 353 that belongs to phenon 3 of *Thornley* fimbriae could only be demonstrated after staining with phosphotungstic acid or uranyl acetate.

Strain 316_r—from *Eiken s* collection of *Bacteroides corrodens* strains—turned out to be a strictly anaerobic, gram-negative, polarly flagellated rod exhibiting twitching motility (11) Electron microscopy showed that this strain had one polar flagellum and polar fimbriae of a slightly smaller diameter (40 Å) than the other fimbriated strains included in this study (Figs. 12 and 13)

The marine bacteria, strains NCMB 247 and 263 are yellow-pigmented, gram-negative rods that produce relatively large spreading zones on SA plates. The micromorphological pattern of their spreading zones and the manner of the cell movements suggest that NCMB 247 spreads by gliding and NCMB 263 by twitching motility (11) Both these strains were examined repeatedly in the electron microscope using different preparation methods for negative staining as well as different negative stains. NCMB 263 was found to possess polar fimbriae (Fig 14) whereas fimbriae were never demonstrated on cells of NCMB 247 (Fig 15)

The results entered in Table 2 demonstrate that gliding motility is unrelated to fimbria-



Fig 16 Vitreoscilla sp. strain UWO 390. "Suspension method" preparation from spreading zones of 20 hour-old colonies on MEA. Small bundles of detached fimbriae are present along the side of this cell.

Fig 17 Vitreoscilla sp. strain UWO 390-R. "Suspension method" preparation from the periphery of 20 hour-old colonies on MEA. These two cells present a few fimbriae close to their surface.

tion since only one of the strains had fimbriae and the same kind of structures was found, although in a smaller number on cells of a variant of this strain without gliding motility (Figs. 16 and 17)

DISCUSSION

Ps echinoides and *Caulobacter* sp. have polar fimbriae with diameters of about 50 Å and 40 Å, respectively (14 20) Such strains have not been examined for twitching motility Polar fimbriae have also been observed on *Ps testosteroni Ps alcaligenes* and *Ps maltophilia* (9) on *Ps. aeruginosa Ps. acidovorans* and *Ps solanacearum* (9 and present study) and on *Ps pseudoalcaligenes Ps putrefaciens* and *Ps stutzeri* (present study) In a study comprising about 250 strains, representing 21 different species of *Pseudomonas* twitching motility was demonstrated only in strains of these nine species (11) The anaerobic polarly fimbriated strains that form spreading colonies, described by *Jackson et al.* (15) also exhibited twitching motility (11)

Apart from the species mentioned above, the other polarly fimbriated strains included in this study strains of *Acinetobacter calcoaceticus* (12) and possibly of *Moraxella bovis* (2) we do not know of other examples of bacteria with polar fimbriae.

Several species of enterobacteria, on the other hand, are known to possess peritrichous fimbriae of different types (5) but none of more than 90 strains of enterobacteria representing 15 species exhibited twitching motility (11)

In the present study a number of different species of gram-negative bacteria exhibiting twitching motility was shown to possess fimbriae thus confirming the previous reports of a correlation between fimbriation and twitching motility in three species of *Moraxella* and in *Acinetobacter calcoaceticus* (12, 13) The fimbriae observed had a polar origin in all cases where the origin could be

determined with certainty The diameter of the fimbriae was approximately 50 Å, with the exception of an anaerobic strain whose fimbriae had a diameter of approximately 40 Å thus the fimbriation was in all respects of the same kind as that observed in *A. calcoaceticus*

The same kind of fimbriae was never found on non-twitching strains or substrains, although sought for repeatedly using what has been shown to be optimal methods (12) and varying conditions as regards media, cultural conditions and the negative stains used

On the whole our results with different species of *Pseudomonas* agree with those obtained by *Fuerst & Hayward* (9) but a few discrepancies should be mentioned We found no fimbriae on cells of our strain of *Ps cepacia* while *Fuerst & Hayward* found their two strains to be peritrichously fimbriated—like the one strain studied by *Tweedy et al.* (22) and the one strain of *Ps putrefaciens* that *Fuerst & Hayward* studied had no fimbriae, whereas we found that one of our two strains of *Ps putrefaciens* had fimbriae (presumably of a polar origin) and the other had none. Our fimbriated strain exhibited twitching motility but only on SA plates that have a concentration of sodium chloride three times as high as the medium used by *Fuerst & Hayward* (11)

Within the limits of the error of determination, the fimbrial diameters found by us agree with those reported by others (1 9 23) Weiss (23) observed that the number of fimbriae reached a maximum in the logarithmic growth phase. In the present study we did not follow the degree of fimbriation through the different growth phases, but we have found previously that the fimbriae of *Acinetobacter calcoaceticus* could only be regularly demonstrated in the logarithmic growth phase (12)

Eikenella corrodens strain 333 (= NCTC 10596) was examined in the electron microscope by *Jackson et al.* (15) using suspensions (in PTA) from culture plates "An occasional process, possibly a pilus, was seen" Likewise we could only demonstrate f... fimbriae on cells of this strain that exhibited twitching motility but none on cells of the non-twitching substrain.

Little is known about the function of polar fimbriae Besides being associated with twitching motility in all species examined, their presence is correlated with competence in genetic transformation and with the ability to colonize the bovine conjunctiva in *Moraxella bovis* (3 19) It has been speculated that the fimbriae might be able to contract or retract (8) and the resemblance of the filaments of *Ps testosteroni* to F-actin filaments has been pointed out (9)

REFERENCES

1 *Bradley D E.*, The structure and infective process of a *Pseudomonas aeruginosa* bacteriophage containing ribonucleic acid. J gen. Microbiol. 45 83–96, 1966.

2. *Bovre K. & Froholm, L. O* Variation of colony morphology reflecting fimbriation in *Moraxella bovis* and two reference strains of *M nonliquefaciens*. Acta path. microbiol. scand. Sect. B *80* 629–640, 1972.

3. *Bovre K. & F holm L. O.*, Competence in genetic transformation related to colony type and fimbriation in three species of *Moraxella*. Acta path. microbiol. scand. Sect. B, *80* 649–659 1972.

4 *Cohen Bazire G., Sistrom, W R. & Stanier R. Y* Kinetic studies of pigment synthesis by non-sulphur purple bacteria. J cell. comp. Physiol. *49* 25–68, 1957

5 *Duguid J P* The function of bacterial fimbriae. Arch. Immunol. Ther exp. *16* 173–188, 1968.

6. *Eiken M* Studies on an anaerobic, rod-shaped, gram-negative microorganism. *Bacteroides corrodens* n.sp. Acta path. microbiol. scand. 43 404–416, 1958.

7 *Eiken M* Undersøgelser over *Bacteroides corrodens*. Thesis, Copenhagen 1960.

8. *F oholm L. O & Bovre K.*: The fimbriated state and competence in genetic transformation of three *Moraxella* species. In: Archer L. J (Ed.) Bacterial Transformation. Academic Press, London 1973 p. 125–139

9 *Fuerst J A & Hayward, A. C* Surface appendages similar to fimbriae (pili) on *Pseudomonas* species. J gen Microbiol. *58* 227–237 1969

10. *H enrichsen J.*, Bacterial surface translocation a survey and a classification. Bact. Rev 36 1972.

1 *Henrichsen J* The occurrence of twitching motility among gram-negative bacteria. Acta path. microbiol. scand. Sect. B, *83* 171–178 1975

2. *Henrichsen J & Blom J* Correlation between twitching motility and possession of polar fimbriae in *Acinetobacter calcoaceticus* Acta path. microbiol scand. Sect. B, *83* 103–115 1975

3 *Henrichsen J Froholm L. O & Bøvre K* Studies on bacterial surface translocation. 2. Correlation of twitching motility and fimbriation in colony variants of *Moraxell nonliquefaciens M bovis* and *M kingii*. Acta path. microbiol. scand. Sect. B, *80* 445–452, 1972.

4 *Heumann W & Marx R.* Feinstruktur und Funktion der Fimbrien bei dem sternbildenden Bakterium Pseudomonas echinoides. Arch. Mikrobiol. 47 325–337 1964

5. *Jackson F L., Goodman Y E. Bel F R., Wong, P C & Whitehouse R. L. S.*. Taxonomic status of facultative and strictly anaerobic "corroding bacilli" that have been classified as *Bacteroides corrodens*. J med. Microbiol. *4* 171–184 1970.

6. *Lautrop H Bacterium anitratum* transferred to the genus *Cytophaga*. Int. Bull. bact. Nomencl. *11* 107–108 1961

17 *McMeekin T A., Patterson, J T & Murray J G* An initial approach to the taxonomy of some gram negative yellow pigmented rods. J appl. Bact. *34* 699–716 1971

18. *Olsen H Flavobacterium meningosepticum* A bacteriological, epidemiological and clinical study Andelsbogtrykkeriet i Odense, Denmark 1969

19 *Pedersen, K. B., Froholm, L. O & Bøvre K* Fimbriation and colony type of *Moraxella bovis* in relation to conjunctival colonization and development of keratoconjunctivitis in cattle. Acta path. microbiol. scand. Sect. B, *80* 911–918, 1972.

20. *Schmidt J M* Observations on the adsorption of caulobacter bacteriophages containing ribonucleic acid. J gen. Microbiol. *45* 347–353 1966.

21 *Thornley M J.* A taxonomic study of *Acinetobacter* and related genera. J gen. Microbiol *49* 211–257 1967

22 *Tweedy J M Park R. W A. & Hodgkiss W* Evidence for the presence of fimbriae (pili) on *Vibrio* species. J gen. Microbiol. *51* 235–244 1968

23 *Weiss R. L.* The structure and occurrence of pili (fimbriae) on *Pseudomonas aeruginosa* J gen. Microbiol. *67* 135–143 1971

Acta path. microbiol. scand. Sect. B 83 171–178 1975

THE OCCURRENCE OF TWITCHING MOTILITY AMONG GRAM-NEGATIVE BACTERIA

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Henrichsen, J. The occurrence of twitching motility among gram-negative bacteria. Acta path. microbiol. scand. Sect. B, 83 171–178, 1975

Almost 1000 strains representing well above 50 different species or groups of gram-negative bacteria were examined for twitching motility. This kind of motility was mainly found in strictly aerobic cocci and rods (viz. *Acinetobacter calcoaceticus*, *Moraxella* spp., *Neisseria gonorrhoeae*, *N. meningitidis*, *Pseudomonas* spp., phenon 3 of Thornley that is closely related to *Acinetobacter* and marine, yellow-pigmented rods) but also in the facultative *Eikenella corrodens* and in anaerobic strains presumptively identified as "*Bacteroides corrodens*" earlier. Strains of species known to possess polar fimbriae were shown to exhibit twitching motility. None of the strains of species known to possess peritrichously arranged fimbriae exhibited twitching motility.

Key words: Twitching motility; gram-negative bacteria.

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Twitching motility was first demonstrated in strains of *Acinetobacter calcoaceticus* in 1961 by *Lautrop* (25). In 1972, I discussed in a review the then existing information, especially in relation to other bacterial surface translocation phenomena (15). At that time this kind of motility was known to occur also in strains of *Moraxella lacunata* (31), *M. bovis*, *M. kingae* and *M. nonliquefaciens* (18, 31), in non-flagellated strains of *Pseudomonas aeruginosa* (26), in an unnamed *Moraxella* sp. (2), and in some gram-negative yellow-pigmented rods (29).

As part of a systematic study of twitching motility a large-scale search for the occurrence of twitching motility among gram-negative bacteria – and to some extent among gram-positive bacteria – was planned. Because it was recently discovered that strains known to exhibit twitching motility possessed polar fimbriae (16, 17), the search for twitching motility was especially directed towards bacteria known to possess this kind of fimbriae.

A number of strains of species known to possess peritrichously arranged fimbriae were already under study, e.g. the enterobacteria, but in order to firmly exclude the possibility that peritrichously arranged fimbriae had any correlation to twitching motility, the search was also extended to comprise other strains known to possess this kind of fimbriae, e.g. *Aeromonas* sp. and *Agrobacterium* spp.

The results of the search for twitching motility are presented in this paper.

Henrichsen J., The occurrence of twitching motility among gram-negative bacteria. Acta path. microbiol. scand. Sect. B *83*: 171–178 1975

Henrichsen J & *Blom, J.*. Correlation between twitching motility and possession of polar fimbriae in *Acinetobacter calcoaceticus*. Acta path. microbiol. scand. Sect. B *83* 103–115 1975

Henrichsen J Froholm L. O & *Bøvre K* Studies on bacterial surface translocation. 2 Correlation of twitching motility and fimbriation in colony variants of *Moraxella nonliquefaciens M bovis* and *M Hagil* Acta path. microbiol. scand. Sect. B *80* 445–452 1972.

Heumann W & *Marx R.* Feinstruktur und Funktion der Fimbrien bei dem sternbildenden Bakterium Pseudomonas echinoides. Arch. Mikrobiol. *47* 325–337 1964

Jackson F L. Goodman Y E., Bel F R., Wong, P C & *Whitehouse R. L. S.*. Taxonomic status of facultative and strictly anaerobic "corroding bacilli" that have been classified as *Bacteroides corrodens*. J med. Microbiol. *4* 171–184 1970.

Lautrop H Bacterium anitratum transferred to the genus *Cytophaga*. Int. Bull. bact. Nomencl. *11*: 107–108 1961

17 *McMeekin T A Patterson, J T* & *Murray, J G.*. An initial approach to the taxonomy of some gram negative yellow pigmented rods. J appl. Bact. *34* 699–716, 1971

18. *Olsen H.*, *Flavobacterium meningosepticum* A bacteriological, epidemiological and clinical study Andelsbogtrykkeriet I Odense, Denmark 1969

19 *Pedersen K. B., Froholm L. O* & *Bøvre, K.* Fimbriation and colony type of *Moraxella bovis* in relation to conjunctival colonization and development of keratoconjunctivitis in cattle. Acta path. microbiol. scand. Sect B, *80* 911–918, 1972.

20. *Schmidt J M*: Observations on the adsorption of caulobacter bacteriophages containing ribonucleic acid. J gen. Microbiol. *45* 347–353 1966.

21 *Thornley M J* A taxonomic study of *Acinetobacter* and related genera. J gen. Microbiol. *49* 211–257 1967

22. *Tweedy J M., Park R. W A.* & *Hodgkiss W* Evidence for the presence of fimbriae (pili) on *Vibrio* species. J gen. Microbiol. *51* 235–244 1968.

23 *Weiss R. L.* The structure and occurrence of pili (fimbriae) on *Pseudomonas aeruginosa*. J gen. Microbiol. 67 135–143 1971

strains cannot. Strain NCMB 11 had 34.2 per cent GC (7)

Moraxella. Three strains of each of the species *M bovis*, *M kingae* and *M nonliquefaciens* have been described before (18) but are mentioned again for the sake of completeness. The supplementary strains of these species as well as most of the strains of *M osloensis* two of the *M phenylpyruvica* strains and the one *M urethralis* (27) strain were isolated from clinical specimens at SSI. The type strain of *M osloensis* (ATCC 19976) and the neotype strain of *M phenylpyruvica* (ATCC 23333) were included in the present study

Neisseria Of the 15 strains of *N gonorrhoeae* included, five were laboratory strains with only colony types 3 and 4 of *Kellogg et al.* (23) and eight were fresh isolates from cases of gonorrhoea. Six of these freshly isolated strains were dominated by colony types 1 and 2, and two were indeterminable as to type. All strains were supplied by *Alice Reyn* the Neisseria Department, SSI who also assisted in the colony typing by the method described by *Jephcott & Reyn* (21) The 24 strains of *N meningitidis* were all freshly isolated from cases of meningitis at the Department of Diagnostic Bacteriology SSI. They were examined for twitching motility after no more than one *in vitro* transfer upon isolation.

Two strains of *Photobacterium phosphoreum* were isolated in the Department of Diagnostic Bacteriology SSI, and one strain was isolated from a ulcer by *W Frederiksen* Department of Medical Bacteriology County Hospital of Aalborg South, Aalborg, Denmark.

Pseudomonas The strains of *Ps. mallei* were supplied by *G A. Hottle* University of California, Naval Biological Laboratory Oakland, California, USA. The strains of *Ps. solanacearum*, 002A, 003B and 017A were isolated and supplied by *A C Hayward* Department of Microbiology Uni ersity of Queensland, Brisbane, Australia All other strains of *Pse domonas* spp. were from the SSI collection

Vibrio All strains were isolated from clinical specimens by *W Frederiksen* Aalborg, Denmark.

One of the three strains of *Thornley's phenon 3* (35) A 333, was isolated at the Department of Diagnostic Bacteriology SSI the two other strains (MJT/F5/153 and MJT/F5/156) were supplied by *Margaret J Thornley* Sub-Department of Chemical Microbiology Department of Biochemistry University of Cambridge, England.

Media

In order to meet the different requirements of the many organisms several kinds of media had to be used they are listed in Table 1 Plates were prepared by pouring 30 ml of the medium into 9 cm Petri-dishes. All media were freshly poured when used and only dried for 20 minutes at 35 C immediately prior to inoculation.

TABLE 1 *Media Used for the Examination of the Different Species*

Medium*	Species or groups of bacteria examined
Cystine-haemin-nutrient agar (20)	*"Bacteroides corrodens"* *Eikenella corrodens*
Salt agar (17)	Marine, gram-negative, *yellow-pigmented rods*
Ascites agar (15)	*Moraxella bovis* *M kingae* *M nonliquefaciens* *Neisseria gonorrhoeae* *N meningitidis*
Kellogg medium (23)	*N gonorrhoeae*
Meat extract agar (15) with 3% sodium chloride	*Vibrio alginolyticus*
Cytophaga agar no. 62 (15) with 0.9% sodium chloride	*Vibrio alginolyticus*
Cytophaga agar no. 62 (15)	All species not already listed

* Numbers in brackets indicate the reference where the description of the medium is given.

Inoculation

Each plate was inoculated with two parallel streaks—of a particular strain—made with small platinum loop.

Incubation

Culture plates were incubated in plastic bags or closed jars with a layer of water in the bottom in order to ensure a humid atmosphere. The temperature used was 30 C for most strains. However the marine, yellow-pigmented rods and the strains of *Thornley's* phenon 3 were incubated at 22 C, and strains of *"Bacteroides corrodens"* *Eikenella corrodens* *Moraxella bovis* *M kingae* *M nonliquefaciens* *Neisseria gonorrhoeae* and *N meningitidis* were incubated at 35 C. Non-twitching strains were usually observed for 7 days before being registered as negative.

Agar Plate Microscopy was carried out as described previously using low-power dry lenses (15) Because some twitching bacteria form spreading zones which are only barely visible even with a hand lens, *e.g. N gonorrhoeae* and *N meningitidis*

TABLE 2. *Species Studied and Results of Search for Twitching Motility*

Species or groups of bacteria studied	Polar fimbriae[a]	Twitching motility[b]
Acinetobacter calcoaceticus	+ (16)	101 (285)
Aeromonas sp.	— (36)	0 (10)
Agrobacteria		
Agrobacterium radiobacter	— (6)	0 (11)
Agrobacterium tumefaciens	— (6)	0 (3)
Agrobacterium sp.		0 (3)
Alcaligenes odorans		0 (6)
"*Bacteroides corrodens*" I)	+ (17)	2 (3)
"*Bacteroides corrodens*" II)	+ (20)	3 (3)
Eikenella corrodens	+ (17)	4 (4)
Enterobacteriaceae	— (8)	
Arizona sp.		0 (3)
Citrobacter sp		0 (3)
Escherichia coli		0 (27)
Edwardsiella tarda		0 (4)
Enterobacter aerogenes		0 (5)
Enterobacter cloacae		0 (10)
Klebsiella oxytoca		0 (5)
Klebsiella pneumoniae		0 (5)
Proteus mirabilis		0 (16)
Proteus vulgaris		0 (11)
Proteus morganii		0 (3)
Proteus rettgeri		0 (1)
Proteus inconstans		0 (7)
Salmonella gallinarum		0 (2)
Salmonella sp.		0 (11)
Shigella sp.		0 (9)
"Flavobacteria		
Flavobacterium meningosepticum[c]	— (17)	0 (86)
Flavobacterium sp.)		0 (63)
Marine gram-negative, yellow-pigmented rods[c]	+ (17)	9 (77)
Moraxella		
Moraxella bovis	+ (4)	3 (3)
Moraxella kingae	+? (11)	4 (4)
Moraxella nonliquefaciens	+? (3)	5 (7)
Moraxella osloensis		8 (36)
Moraxella phenylpyruvica		0 (3)
"*Moraxella urethralis*" (27)		0 (1)
Neisseria		
Neisseria gonorrhoeae	(+) (22 34)	5 (13)
Neisseria meningitidis	(+) (12)	9 (24)
Photobacterium phosphoreum		0 (3)
Pseudomonas		
Pseudomonas aeruginosa	+ (5 13 17 37)	17 (20)
Pseudomonas fluorescens	— (13)	0 (22)
Pseudomonas putida	— (13 17)	0 (11)
Pseudomonas sp. (fluorescent)		0 (1)
Pseudomonas acidovorans	+ (13 17)	8 (10)
Pseudomonas alcaligenes	+ (13)	7 (8)
Pseudomonas cepacia	— (13 17 36)	0 (9)
Pseudomonas diminuta		0 (10)
Pseudomonas flava		0 (7)
Pseudomonas fragi	— (13)	0 (7)
Pseudomonas mallei		0 (15)
Pseudomonas maltophilia	+ (13)	5 (10)

TABLE 2. (continued)

Species or groups of bacteria studied	Polar fimbriae[a])	Twitching motility[b])
Pseudomonas pickettii (32)		0 (10)
Pseudomonas pseudoalcaligenes	+ (17)	41 (64)
Pseudomonas putrefaciens	+ (17)	3 (8)
Pseudomonas solanacearum	+ (13 17 24)	3 (3)
Pseudomonas solanacearum, assumed biotype of		2 (9)
Pseudomonas stutzeri	+ (17)	13 (21)
Pseudomonas testosteroni	+ (13)	5 (6)
Pseudomonas vesicularis		0 (2)
Pseudomonas sp., unnamed taxon		0 (4)
Vibrio		
Vibrio alginolyticus		0 (3)
Unnamed species		
Phenon 3 of *Thornley* (35)	+ (17)	1 (3)

a) + = polar fimbriae have been observed on one or more strains of the species It was not always the same strains which were examined for twitching motility during the present study — = polar fimbriae have not been *found on strains of the species, but peritrichous fimbriae may or may not have* been found +? = the origin of the fimbriae was not definitely determined, but it is most likely polar (see ref. 16); (+) = fimbriae on cocci with more than one plane of division can hardly be "polar" but these fimbriae *resemble* polar fimbriae (see ref. 16) Numbers in brackets refer to the list of references.

b) The total number of strains found to exhibit twitching motility is listed for each species. The numbers in brackets indicate the total number of strains examined.

c) See *Materials and Methods* for comments on the systematics of these taxa.

all strains were examined under the microscope, regardless of the macroscopic appearance of growth. The culture plates were as a rule examined after 1 3 and 7 days of incubation. Microscopically the spreading zones were evaluated and differentiated according to previously given definitions (15) Occasionally an oil immersion objective (× 100) was also used, but motility which could only be seen after the application of a cover slip was not registered in this study because of the uncertainty in evaluating the kind of process going on in this rather different system.

RESULTS

The results are listed in Table 2 together with available information, compiled from the literature, about polar fimbriae on strains of the species examined.

Only few comments are needed about some of the species

Acinetobacter calcoaceticus Twitching motility occurred with an approximately 50 per cent higher frequency among strains that produced carbohydrates than among strains that did not produce acid, *viz* 45 per cent and 28 per cent, respectively

"Bacteroides corrodens" II and *Eikenella corrodens* The non-spreading substrains did not exhibit twitching motility

The following strains of the *marine gram negative yellow-pigmented rods* were judged to display twitching motility NCMB 245 246, 252, 255 258, 261 263 273 and 298. However these results are to be considered with some reservation because the evaluation of all the spreading NCMB strains was difficult (see *Discussion*)

N gonorrhoeae All the five twitching strains were from cases of gonorrhoea and were dominated by colonies of types 1 and 2 of *Kellogg* (23) The spreading zones produced by strains of this species and by strains of *N meningitidis* were almost invisible, even when viewed through a hand lens, with the exception of one strain of *N meningitidis* with bigger zones.

During the study other kinds of bacterial

TABLE 3 *Kinds of Surface Translocation Other than Twitching Motility Observed*

Kind of surface translocation	Bacterial species or group	Number of strains with spreading growth[a]
Gliding	Marine gram-negative, yellow-pigmented rods[b] (NCMB 247 256, 257 272, 275 and 11)	6 (27)
Sliding	*Acinetobacter calcoaceticus*	?[c]
	Alcaligenes odorans	6 (6)
	Flavobacterium meningosepticum	55 (96)
	Flavobacterium sp.	9 (65)
	Moraxella phenylpyruvic	1 (3)
	Pseudomonas cepacia	6 (9)
	Pseudomonas pickettii	6 (10)
Swarming	*Vibrio alginolyticus*[d]	3 (3)
Swimming	*Aeromonas* sp.	2 (10)
	Agrobacterium spp.	12 (17)
	Pseudomonas fluorescens	
	biotype A of *Stanier et al.* (33)	5 (5)
	biotype G of *Stanier et al.* (33)	4 (5)

a) Numbers in brackets indicate the total number of strains examined.
b) See *Discussion*.
c) Sliding strains of this species were encountered but not registered systematically throughout the study
d) In keeping with the findings by *Baumann et al.* (1) and the definition of swarming given previously (15) these strains showed heavy peritrichous flagellation when swarming.

Surface translocation than twitching motility were also observed. These are listed in Table 3

DISCUSSION

The first aim of this study was to shed light on the occurrence of twitching motility among gram-negative bacteria. Obviously the list of species studied is far from complete. However almost 1000 strains representing well above 50 species were examined and new information was gained So far twitching motility was known to occur only among species of aerobic, gram-negative rods. Now it has been demonstrated that other taxa may display this kind of motility too viz. anaerobic, polarly flagellated, gram-negative rods (listed as "*Bacteroides corrodens*" I in Table 2) anaerobic, non-flagellated gram-negative rods (listed as "*Bacteroides corrodens*" II in Table 2) *Eikenella corrodens Neisseria gonorrhoeae* and *N meningitidis* Twitching motility was also revealed in some additional species of aerobic, gram-negative rods, notably *Pseudomonas* spp Until quite recently twitching had not been observed among gram-positive bacteria, but in a study by *Henrichsen & Henrichsen* (19) it was found that the spreading zones produced by strains of *Streptococcus sanguis* were due to twitching and that these bacteria possess polar fimbriae. In consequence it can now be stated that twitching occurs in both gram-negative and gram-positive bacteria, and in both strictly aerobic, facultatively and strictly anaerobic organisms.

A second aim was to find out whether strains of species known to possess polar fimbriae also exhibited twitching motility since all strains with twitching motility examined so far had been shown to possess polar fimbriae (16, 17) This correlation was definitely confirmed by the present study Not only were new examples of the correlation demonstrated, but among a large number of species known to possess peritrichously arranged fimbriae twitching motility was never

ound. Examples of such species are *Aeromonas* spp. (36) *Agrobacterium* spp. (6) any species of enterobacteria (8) *Pseudomonas cepacia* (13 36) and *Ps fragi* (13)

The evaluation of the kind of surface trans-ocation performed by strains of the marine, n-negative yellow-pigmented rods with preading growth was difficult. As will be n from Table 2 and 3 some of the strains re recorded as performing twitching and hers as performing gliding motility In trains recorded as twitching, the micro-orphological pattern of the spreading zones was typical of twitching but some individual lls exhibited a fairly steady gliding. In rains recorded as gliding, the cells did glide, ut the micromorphological pattern of the preading zones was not entirely typical be ause the formation of rafts and spearheads as not very pronounced, so that more cells oved singly than is usually observed in ading, thereby approaching the pattern of itching. The difficulty in differentiating en gliding and twitching motility in hese strains, therefore leaves it a moot point hether the two kinds of surface transloca ion were correctly determined. Twitching otility was found exclusively among strains onging to group I of *Hayes* (14) which, ccording to DNA analysis, cannot be cyto-agas, and gliding motility was mainly found strains of group 4 which could be cyto-nagas. One strain of group 1 (NCMB 263) as been found previously to possess polar brine and one strain of group 4 (NCMB 47) had no fimbriae (17) In view of the rmly established correlation between twitch-g motility and possession of polar fimbriae, e non-fimbriated strain NCMB 247 does in l probability perform gliding motility On e other hand, as fimbriae have previously en observed in a definitely gliding strain, e possession of polar fimbriae by strain CMB 263 does not necessarily lead to the nclusion that the motility of this strain is the twitching kind, even though this seems be the most likely possibility

Pu hand (personal communication 1967) s stated that all strains of *A calcoac t us*

examined by him showed twitching motility I found an overall twitching rate of 35 per cent among such strains. If the twitching motility that could only be observed after application of a coverslip was included, the figure was approximately 50 per cent. *Piechaud* used an oil chamber in which the bacteria are situated in a thin fluid layer interposed between agar and oil (31) The reason why this method is more sensitive than direct agar plate microscopy could be that in the oil chamber the conditions are so different that even strains which lack polar fimbriae may thanks to the possession of other surface properties that are presumably required exhibit twitching motility

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I thank all those, mentioned in *Materials and Methods* who supplied me with strains. Special thanks are due to *Alic Reyn* for assistance in the evaluation of the colony types of *Neisseria gener hoeae*

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REFERENCES

1 *Baumann P Baumann L & Reichelt J L.* Taxonomy of marine bacteria *Ben has parahaemolytica* and *Beneckea alginolytica*. J Bact. 113 1144–1155 1973

2 *Bijlsterveld O P van* New *Moraxella* strain isolated from angular conjunctivitis. Appl Microbiol 20 405–408 1970

3 *Bøvr K Bergan T & Frøholm L O* Electron microscopical and serological characteristics associated with colony type in *Moraxella nonliquefaciens* Acta path. microbiol. scand. Sect B, 78 765–779 1970.

4 *Bøvre K & Frøholm L O* Variation of colony morphology reflecting fimbriation in *Moraxella bovis* and two reference strains of *M nonliquefaciens*. Acta path. microbiol. scand. Sect. B, 80 629–640 1972.

5 *Bradley D E.* The structure and infective process of a *Pseudomonas aeruginosa* bacteriophage containing ribonucleic acid. J gen. Microbiol 45 83–96 1966.

6 *De Ley J Bernaert M Rassel, A & Guilmot J* Approach to an improved taxonomy of the genus *Agrobacterium* J gen. Microbiol. 43 7 17 1966.

7 *De Ley J & van M ylem J* Some applications of deoxyribonucleic acid base composition in bacterial taxonomy Antonie v Leeuwenhoek 29 344 358, 1963.

8. *Duguid J P* The function of bacterial fimbriae. Arch. Immunol. Ther exp *16* 173–188, 1968.

9 *Eiken M* Studies on an anaerobic, rod-shaped, gram-negative microorganism *Bacteroides corrodens* n.sp. Acta path. microbiol. scand. *43* 404–416 1958.

10 *Eiken M* Undersogelser over *Bacteroides corrodens*. Thesis, Copenhagen 1960

11 *Froholm L. O & Bovre K* Fimbriation associated with the spreading-corroding colony type in *Moraxella kingii*. Acta path. microbiol. scand. Sect. B, *80* 641–648, 1972.

12 *Froholm L. O Jyssum, K & Bovre K.* Electron microscopical and cultural features of *Neisseria meningitidis* competence variants. Acta path. microbiol scand. Sect. B, *81* 525–537 1973

13. *Fuerst J A & Hayward A C* Surface appendages similar to fimbriae (pili) on *Pseudomonas* species. J gen. Microbiol. *58* 227–237 1969

14 *Hayes P R.* Studies on marine flavobacteria. J gen. Microbiol. *30* 1–19 1963

15 *Henrichsen J* Bacterial surface translocation a survey and a classification. Bact. Rev. *36* 478–503 1972.

16. *Henrichsen J & Blom J* Correlation between twitching motility and possession of polar fimbriae in *Acinetobacter calcoaceticus*. Acta path. microbiol. scand. Sect. B *83* 103–115 1975

17 *Henrichsen J & Blom, J* Examination of fimbriation of some gram-negative rods with and without twitching and gliding motility Acta path. microbiol. scand. Sect. B, *83* 161–170 1975

18. *Henrichsen J Froholm L. O & Bovre K* Studies on bacterial surface translocation. 2. Correlation of twitching motility and fimbriation in colony variants of *Moraxella nonliquefaciens M bovis* and *M kingii*. Acta path. microbiol. scand. Sect. B, *80* 445–452, 1972.

19 *Henriksen S D & Henrichsen J* Twitching motility and possession of polar fimbriae in spreading *Streptococcus sanguis* isolates from the human throat. Acta path. microbiol. scand. Sect. B, *83* 133–140 1975

20. *Jackson F L, Goodman Y E Bel F R Wong, P C & Whitehouse R. L. S* Taxonomic status of facultative and strictly anaerobic corroding bacilli" that have been classified as *Bacteroides corrodens* J med. Microbiol. *4* 171–184 1970.

21 *Jephcott A E. & Reyn A* *Neisseria gonorrhoeae* Colony variation I Acta path. microbiol. scand. Sect. B, 79 609–614 1971

22. *Jephcott A E. Reyn A & Birch-Andersen A* *Neisseria gonorrhoeae* III. Demonstration of presumed appendages to cells from different colony types. Acta path. microbiol. scand. Sect. B, *79* 437–439 1971

23 *Kellogg D S Jr Peacock W L, Jr Deacon, W E. Brown, L. & Pirkle C. L* *Neisseria gonorrhoeae* I Virulence genetically linked to clonal variation. J Bact. 85. 1274–1279 1963

24 *Kelman A & Hruschka, J* The role of motility and aerotaxis in the selective increase of avirulent bacteria in still broth cultures of *Pseudomonas solanacearum*. J gen. Microbiol. *76* 177–188 1973

25 *Lautrop H* *Bacterium anitratum* transferred to the genus *Cytophaga*. Int. Bull bact. Nomencl. *11* 107–108, 1961

26 *Lautrop H* Gliding motility in bacteria as a taxonomic criterion. Publ. Fac. Sci. Univ. J E. Purkyne, Brno, Ser K, *35* 322–327 1965

27 *Lautrop H., Bovre K. & Frederiksen, W* A *Moraxella*-like microorganism isolated from the genito-urinary tract of man. Acta path. microbiol scand. Sect. B, *78* 255–256, 1970

28. *Mandel, M & Leadbetter E. R.* Deoxyribonucleic acid base composition of myxobacteria. J Bact. *90* 1795–1796 1965.

29 *McMeekin T A Patterson, J T & Murray J G* An initial approach to the taxonomy of some gram negative yellow pigmented rods J appl. Bact. *34* 699–716 1971

30. *Olsen H.* *Flavobacterium meningosepticum* A bacteriological, epidemiological and clinical study Thesis, Andelsbogtrykkeriet, Odense 1969

31 *Piéchaud M* Mobilité chez les *Moraxella*. Ann. Inst. Pasteur *104* 291–297 1963.

32. *Ralston E. Palleroni N J & Doudoroff M* *Pseudomonas pickettii* new species of clinical origin related to *Pseudomonas solanacearum* Int. J system. Bact. *23* 15–19 1973.

33 *Stanier R. Y Palleroni N J & Doudoroff M* The aerobic pseudomonads a taxonomic study J gen Microbiol. *43* 159–271 1966

34 *Swanson J., Kraus S J & Gotschlich, E C* Studies on gonococcus infection. I. Pili and zones of adhesion: Their relation to gonococcal growth patterns. J exp. Med. *134* 886–906, 1971

35. *Thornley M J* A taxonomic study of *Acinetobacter* and related genera J gen. Microbiol. *49* 211–257 1967

36. *Tweedy J M Park R W A & Hodgkiss W* Evidence for the presence of fimbriae (pili) on *Vibrio* species J gen. Microbiol *51* 235–244 1968.

37 *Weiss R L. & Raj H D* The structure and isolation of pili (*fimbriae*) of *Pseudomonas aeruginosa*. Aust. J exp. Biol. med. Sci. *50* 559–566 1972.

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THE INFLUENCE OF CHANGES IN THE ENVIRONMENT ON TWITCHING MOTILITY

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Henrichsen, J The influence of changes in the environment on twitching motility Acta path. microbiol. scand. Sect. B, 83 179–186, 1975

By examining medium composition and cultural conditions quantitatively it was possible to define conditions suitable to bring twitching motility about. Such conditions include the use of freshly poured, relatively thick and only slightly dried plates of a dilute medium in which the agar concentration is not too high. Incubation should take place in a humid atmosphere. In fact, everything points to the humidity as a factor of the utmost importance. Using strains of *Acinetobacter calcoaceticus* it was found that a pH value of the medium adjusted to 9.0 enhances twitching motility. Spreading growth was not produced if agarose was applied instead of ordinary agar. Twitching motility was inhibited by a number of different chlorides, potassium nitrate, Tween 80 and sodium taurocholate. Possible interpretations of these observations are discussed.

Key words Twitching motility environment

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Twitching motility is a special kind of bacterial surface translocation it may lead to the production of spreading growth on media poor in nutrients, but usually this will not happen on ordinary laboratory media (3) Although the first description of this kind of surface translocation appeared in 1961 (8) there is still only very little information about optimal experimental conditions to be found in the literature A medium poor in nutrients and with an agar concentration of 0.9 per cent was described by *Halvorsen* (2) but he did not examine other media. *Pitchaud* (10) who studied twitching motility in a specially designed oil chamber considered an agar concentration of 0.5 to 0 7 per cent superior to higher concentrations. *Gianelli & Cabasso* (1) using Difco agar recommended the same concentration they also reported that higher concentrations of nutrients gave better results with some strains than the concentrations used by *Halvorsen* and that varying the pH from the original 7 2, altering the surface tension by addition of Tween 80 and using other energy sources did not enhance the spreading of their strains.

Since only this scanty information was available, it was deemed necessary to examine systematically the composition of media and cultural conditions in order to define as far as possible the *conditions favourable* for bringing twitching motility to light. In an attempt to approach the question of the mechanism by elucidating *conditions essential* for this kind of motility I have also examined the influ-

ence of changes in the ionic strength and surface tension of the medium brought about by adding different salts and surface active agents in various amounts.

MATERIALS AND METHODS

Bacterial Strains

Five strains of *Acinetobacter calcoaceticus* were used as test strains, one without twitching motility strain A 79 and four with pronounced twitching motility strains ATCC 17905 A 16 A 135 and AB 156. The non-twitching strain has previously been shown to be devoid of polar fimbriae, whereas the four twitching strains possess polar fimbriae (5)

Agar Plate Microscopy was carried out as described before (3)

Experimental Technique

A modified cytophaga agar (medium no. 62) and a defined set of experimental conditions were chosen as starting point (basic medium and basic cultural conditions) From this basic situation, stepwise variations were introduced, one by one, as regards medium constituents, medium production, cultural conditions and experimental procedure. Colony diameters were used as an approximate expression of twitching activity and, in each particular experiment, diameters of 7 to 9 hour-old colonies of each strain were measured (see later) and compared with diameters obtained on the set of basic medium plates—kept under basic cultural conditions—which were included in all experiments. The non-twitching strain was also included in all experiments as a control of possible growth inhibitory effects. If the measured colony diameters of the non-twitching strain on a particular set of plates were the same within the experimental error (see below) then the difference between the diameters of each of the twitching strains, measured on the experimental plates and measured on plates with the basic medium kept under the basic cultural conditions, was taken as a measure of the difference in twitching activity under the given circumstances.

In the presence of many colonies on a plate, the final diameter attained by any one colony varies inversely with the number of colonies on the plate (6) As it was desirable to measure more than one colony it was necessary to perform the measurements so early that growth had not been affected by neighbour colonies, i.e. by incipient depletion of nutrients or accumulation of metabolites. The lag of the strains employed was determined to be less than 60 minutes and the generation time during exponential growth was found to be approximately 40 minutes in the liquid equivalent of the basic medium (see later) After 5 hours, each colony should thus consist of at least 100 cells. As twitching motility does not start in colonies until they consist of 50–100 cells (4) it was decided to "read the plates" i.e. measure the colony diameters, after 7 to 9 hours of incubation, when the colonies would consist of more than 1000 cells and twitching motility had taken place for some time. At this time, most colonies of the twitching strains on the basic medium and under basic cultural conditions could be seen to consist entirely of actively twitching cells loosely scattered over the area of the agar surface corresponding to the colony as shown in Fig. 1 of *Henrichsen & Blom* (5)

Basic Medium

As basic medium was used a modified cytophaga agar medium no. 62 made of 0.05 per cent tryptone (Difco) 0.05 per cent yeast extract (Difco) and 1 per cent Bacto agar (Difco) with a final pH adjusted to 7.0 The contents of sodium, potassium and calcium in the basic medium, without any salts added, was determined in a flame-spectrophotometer after incineration by the Substrate Department, *Statens Seruminstitut*, and were found to be approximately 5×10^{-4} M, 6×10^{-4} M and 2×10^{-4} M, respectively

Basic Cultural Conditions

Plates were poured with 25 ml of medium in Petri-dishes with a diameter of 9 cm and were either used immediately or stored in sealed plastic bags, usually not for more than 24 hours. Before inoculation, plates were dried in the inverted position, with the lids removed, in an incubator at 35 C for 30 minutes upon which the droplets of condensate on the Petri-dish lid had disappeared. The plates were then inoculated by flooding them with a suitably diluted 16 hour-old liquid culture (liquid equivalent of medium no. 62) so as to obtain approximately 1000 colonies per plate (a substantially smaller number of colonies per plate made it too time-consuming to find the colonies under the microscope) Excess of the fluid culture was sucked off by a Pasteur pipette and the plates were left on the bench to dry for 5 minutes before incubation in plastic bags at 30° C.

Determination of Colony Diameters

After 7 to 9 hours of incubation the plates were removed from the incubator all plates of one strain at a time. Each plate was then placed on the microscope stage with the co-ordinate indicator in a given position and the microscope was focused on the agar surface. The stage was pushed from left to right, away from the observer from right to left etc., following a specified pattern, until 10 colonies had been encountered, their diameters measured and an opinion about twitching motility

noted. A × 16 dry lens objective was used with a pair of × 12.5 oculars one of which was fitted with a grating with a distance of approximately 31 µm (as viewed in the microscope) between single lines. Diameter were always measured as number of frames of the grating and multiplied by 10, but not converted into µm. The median value of the 10 measured diameters, d was used as the result.

The reading of a set of plates with one strain seldom took more than 15–20 minutes, and the plate with the expectedly biggest colonies was read first.

Significance Limits

In a number of separate experiments, the standard deviation was determined by measuring for each strain the diameters of 250 colonies, i.e. 50 colonies on five plates. The standard deviation, s, was dependent on the level, $\bar{x}$, of the measured diameters, thus $s \sim 0.3\,\bar{x}$. In nearly all the experiments reported in this paper both the mean and the median values were calculated and found to be in good agreement. Median values were, however used in order not to rely too heavily on an assumption of a normal distribution. It was found that, if two values of d (median values) were to be significantly different at the 5 per cent level, the larger value of the two d should be more than 1.4 times the smaller value d_s, i.e. $d_l/d_s > 1.4$* and possibly less if systematic differences were encountered.

...ermination of Generation Time

For each strain, curves were obtained of the growth in the liquid equivalent of the basic medium and also in some modifications of this medium as well as with different concentrations of calcium chloride added. 5 ml of a 16 hour culture inoculated into 50 ml liquid medium incubated in a water bath at 30 C and aerated with atmospheric air. The extinction was followed at intervals of 15 minutes in a Zeiss spectrophotometer. When the extinction was just below 0.5 5 ml of this culture was used to inoculate 150 ml of preheated medium. In this culture the extinction was followed until the bacteria had entered the stationary phase of growth.

In some experiments viable counts were also ... at regular intervals by plating after suitable dilutions. Growth curves obtained both ways were ...formable ... the multiplication rate and the growth rate (doublings in bacterial protoplasm) ... the same. Growth curves of strain A 16 were not obtained as this strain did not grow diffusely in the liquid medium. The four other strains all had generation times between 37 and 40 minutes, under the conditions described.

Gel Strength was measured by the method of *Møller* (9)

RESULTS

Variations of the Basic Medium

1. *Tryptone* The following concentrations were compared (per cent, w/v) 0.01 0.05 (basic medium) 0.1 0.5 1.0, 2.0 4.0 and 5.0. The non-twitching strain A 79 grew equally well on all the plates. Growth experiments gave the same generation times whether 0.1 or 0.5 per cent tryptone was used. As regards the twitching strains there were non-significant differences on plates with the lower concentrations, but above 1.0 per cent, only strain ATCC 17905 showed some twitching activity on plates with 2.0 per cent and no activity on 4.0 per cent plates. If diameters on plates with 1 per cent and 2 per cent were compared, the following d(1 per cent)/d(2 per cent)-values were found in the case of strains A 16 A 135 AB 156 ATCC 17905 and A 79 (non-twitching) 2.8, 3.3 3.4 1.5 and 1.0. It may be concluded that tryptone concentrations above 1.0 per cent inhibit twitching motility under the basic cultural conditions described.

2. *Yeast extract* It applies to all strains that differences between the following concentrations (per cent, w/v) were not found 0.005 0.01 and 0.05 (basic medium).

3. *Agar* The following concentrations of Difco's Bacto agar were examined (per cent, w/v) 0.6 1.0 2.0 3.0 and 4.0. Most strains had insignificantly smaller colonies on plates with 0.6 per cent agar than on plates with 1.0 per cent and microscopy revealed that the bacteria to some extent had sunk down into the agar. The non-twitching strain grew with slightly larger colonies on plates with an agar concentration of 2 per cent (Fig. 1). The twitching strains behaved differently. Strain A 135 (Fig. 1) had the biggest colonies on plates with 1 per cent agar and grew with colonies of gradually decreasing size on plates with higher agar concentrations on 3 per cent agar only little twitching activity was

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* The statistical calculations were carried out by ...s Olsen Larsen Department of Biostatistics, Statens Seruminstitut.

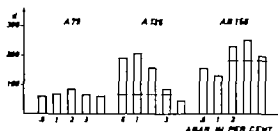


Fig 1 Basic medium with different concentrations of agar Bars on strain A 135 columns refer to comparison with the 4 per cent value, while bars on strain AB 156 columns refer to comparison with the 1 per cent value.

All figures ordinates indicate colony diameters in arbitrary units (see *Materials and Methods*) Bars, if present, indicate level of significance limit.

left and on 4 per cent agar the colonies were quite compact without any twitching Comparing 1 0 per cent with 4 0 per cent, the difference was highly significant (d(1 per cent)/d(4 per cent) = 4.5) Strain A 16 followed the same pattern of variation, although differences were not quite as pronounced (d(1 per cent)/d(4 per cent) = 2 4) Strain AB 156 (Fig 1) behaved differently as it had significantly larger colonies on 2 per cent, 3 per cent and 4 per cent than on 1 per cent agar plates, the largest colonies being in evidence on 3 per cent agar plates (d(2 per cent)/d(1 per cent) = 1 8 and d(3 per cent) /d(1 per cent) = 1.9) On 1 per cent, 2 per cent and 3 per cent agar plates, twitching activity was pronounced. On 4 per cent plates, colonies were smaller though insignificantly than on 3 per cent plates and twitching activity was not as pronounced. Strain ATCC 17905 was excluded for technical reasons.

4 *Different agars* A number of different agars were compared, *viz* agarose from Serva (0.5 per cent) agarose from Mann (0.35 per cent) Ionagar from Oxoid (0.8 per cent) Noble agar from Difco (1 0 per cent) Bacto agar from Difco (1 0 per cent) and Danish agar (1.5 per cent) They were used in the concentrations given in brackets, to give identical gel strengths. The two agaroses are highly purified products in which only few charged groups are left on the molecule and therefore have a very low electro-endosmosis; Bacto agar and Danish agar have a comparatively high electro-endosmosis and Ionagar and Noble agar range somewhere in between (7) The non-twitching strain as well as a randomly chosen strain of *Escherichia coli* grew with larger colonies on Bacto and Danish agar plates than on plates with the purified agar types. Generally twitching *motility was most pronounced on the Bacto* agar plates with broad spreading zones. On plates with the agaroses there was no spreading growth at all but twitching motility was evident inside the colonies. The following d(Bacto agar) / d(Servac agarose)-values were obtained 1.3 (non-twitching strain A 79) 1 6 *(E coli)* 2.8 (ATCC 17905) 2.7 (A 16) 1 8 (A 135) and 4 8 (AB 156) On the plates with the other types of agar some spreading growth and some twitching motility were evident.

5 *pH* Results obtained with strains on plates with a final pH adjusted to 6.0, 7.0, 8 0 and 9 0 are shown in Fig 2. None of the strains grew at pH 5 0 The growth of all strains was inhibited at pH 6.0. Strains AB 156 and ATCC 17905 had the largest colonies and exhibited the most pronounced *twitching motility at pH 9 0 (the* bars on the pH 9 0 columns of these two strains denote values that are 1 4 times as large as the d-values found at pH 7 0 The figure also indi-

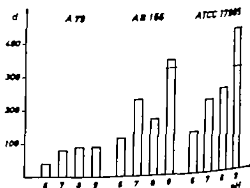


Fig 2 Basic medium with different hydrogen ion concentrations. Bars refer to comparisons with the pH 7.0 value.

0.5 per cent tryptone and was poured in 30 ml amounts.

Monovalent salts were examined in concentrations of 10^{-3} 10^{-2} 10^{-1} and 1 M and divalent salts, as well as salts of higher valencies, in concentrations of 10^{-4} 10^{-3} 10^{-2} and 10^{-1} M, while carbohydrates and urea were examined in concentrations of 10^{-} 10^{-1} and 1 M.

Some chemicals were growth inhibiting without affecting twitching motility at subinhibitory concentrations. These chemicals were the following (the highest concentrations that did not inhibit growth are given in brackets) sodium sulphate (10^{-2} M) sodium acetate (10^{-} M) barium chloride (10^{-2} M) zinc sulphate (10^{-4} M) aluminium sulphate (10^{-4} M) arabinose (10^{-2} M) glucose (10^{-1} M) lactose (10^{-1} M) glycerol (10^{-1} M) and urea (10^{-1} M)

Rodalon® and sodium laurylsulphate inhibited growth in concentrations of 0 05 per cent (w/v) and were not tried in lower concentrations.

Some chemicals, at a particular concentration inhibited spreading (*i.e* colonies were significantly smaller) without affecting growth (*i.e* colony sizes of the non twitching strain) but were growth inhibitory at higher concentrations. Twitching motility of strains ATCC 17905 and A 16 were, however in some cases not affected. These chemicals were the following (the concentrations at which twitching motility but not growth, was affected are given in brackets) potassium chloride (10 M ATCC 17905 and A 16 unaffected) sodium chloride (10^{-} M ATCC 17905 unaffected) ammonium chloride (10^{-1} M ATCC 17905 only insignificantly affected) potassium nitrate (10^{-} M ATCC 17905 only insignificantly affected) calcium chloride (10 M ATCC 17905 and A 16 unaffected) and magnesium chloride (10^{-2} M ATCC 17905 and A 16 unaffected) Although the colonies of the strains affected by these chemicals were without spreading zones, twitching motility was most often seen to occur inside the compact colonies. In Fig 5 examples of results obtained if sodium chlor

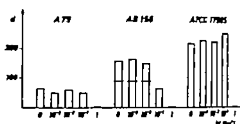


Fig 5 Slightly modified basic medium with diff[er]ent concentrations of sodium chloride added. Ba[rs] on strain AB 156 columns refer to comparison w[ith] the 10^{-} M value.

ide was added to the medium are show[n] There was no inhibition of growth at a co[n]centration of 10^{-1} M, but colonies of stra[in] AB 156 were significantly smaller at this co[n]centration while colonies of ATCC 179[05] were unaffected. The same pattern is se[en] when calcium chloride was added, as show[n] in Fig 6. Growth was inhibited at a concen[]tration of 10^{-1} M but not at 10^{-2} M. Th[is] concentration reduced significantly colon[y] sizes of strain AB 156 while ATCC 17905 a[t] this concentration had significantly bigger colonies. Strain AB 156, and possibly the non twitching strain A 79 also had bigger colonies at a concentration of 10^{-3} M suggesting a stimulation of growth which was confirmed in growth experiments.

Finally sodium taurocholate (Fig. 7) and Tween 80 significantly reduced colony size of twitching strains without inhibiting growth at a concentration of 0 05 per cent (w/v).

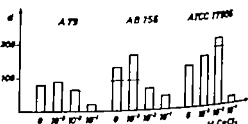
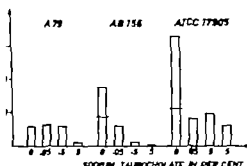


Fig 6 Slightly modified basic medium to which different concentrations of calcium chloride are added Bars on strain AB 156 columns refer to comparison with the 10 M value. Bar on strain ATCC 17905 column refers to comparison with the basic medium alone.



g 7 Slightly modified basic medium to which fferent concentrations of sodium taurocholate are lded Bars refer to comparison with 0.05 per cent dose.

gain no spreading zones were produced at is concentration, but twitching motility was vident in the periphery of the colonies.

DISCUSSION

t is apparent from the results presented that most important factor for the exhibition of witching motility is the amount of liquid vailable at the agar surface. Thus twitching notility was most pronounced on thick plates nly slightly dried, and it was rarely seen if he plates were not incubated in a humid tmosphere. Tryptone concentrations above 1 per cent were found to inhibit twitching motility and this conceivably again reflects the importance of liquid because more free liquid must be available in dilute media. *Stanier* (11) likewise found that a peptone concentration of 2.0 per cent or above would inhibit gliding motility of cytophagas. Agar concentration should also not be too high and for most strains 1–2 per cent was optimal, in contrast to the findings by other investigators according to whom 0.5–0.7 per cent gave the best results (1 10) However their results are not comparable with mine because *Prêchaud* (10) studied twitching motility in an oil chamber and *Gianelli & Cabani* (1) studied it, not only on the agar surface but also between the agar and the bottom of the Petri dishes.

The kind of agar used is clearly of importance for the manifestation of twitching motility Difco's Bacto agar was better than any of the other agars tried. Both of two types of agarose prevented completely the formation of spreading zones. The explanation of this is not clear but it could be of importance that very few charged groups are present in agarose—on the assumption that repulsion between the negatively charged substrate surface and the negatively charged cell surface is involved in the mechanism of twitching During inoculation of the agarose plates it was noted that they were difficult to float, i.e. the applied amount of liquid culture did not spread nearly as readily on the agarose surfaces as on other agar surfaces. This might have a direct bearing on twitching motility which takes place in a thin layer of liquid surrounding the bacterial colony (4) because this film of liquid must of necessity be able to spread with relative ease over the agar surface, if spreading zones are to be produced.

In contrast to plates with the agaroses, plates with Tween 80 and sodium taurocholate were extremely easy to float the applied liquid flattened out immediately on the agar surface which is in keeping with the fact that the liquid phase of such plates has a reduced surface tension. Accordingly the layer of liquid surrounding the colonies might never become sufficiently thick for twitching motility to occur Sodium taurocholate does not inhibit twitching by impairing the fimbriation of twitching strains because electron microscopy showed that cells of strain A 155 possess polar fimbriae also if grown on the basic medium with sodium taurocholate added to a concentration of 0.05 per cent (w/v)

After direct observation of the twitching process, and considering the available information about this process, one easily forms the opinion that it might be under the influence of—and even could be produced by—changes in the electrokinetic potential of the cells an opinion which has been strengthened since it became clear that motility does not take place in direct contact with the substrate surface but in a film of liquid covering the

surface. As the electrokinetic potential is partly dependent on the ionic strength of the environment of the cells it lay near at hand to examine the effect on twitching motility of adding varying concentrations of different ions to the medium. Unfortunately such experiments did not give clear-cut results. Some results were inconclusive because the added ions were growth inhibitory—an insurmountable difficulty if the activity is to be estimated by the size of colonies. Others were partly inconclusive because the measurable effect was not the same on all strains and thus, no general trend was detected However in the experiments with sodium chloride and calcium chloride among others, strain AB 156 provided evidence indicating that the activity was reduced when the ionic strength rose. It is too early to evaluate the significance of this observation it might point to the importance of the electrokinetic potential in the process, but more observations are necessary preferably using a method that is independent of the influence of ions—inhibitory or enhancing—on the growth rate.

REFERENCES

1 *Gianelli F & Cabassi E.* Sciamatura di *Moraxella.* Igiene mod. *40* 81 90, 1967
2. *Halvorsen J F :* Gliding motility in the organisms *Bacterium ambitatum* (B5W) *Moraxella bovis* and *Alcaligenes haemolysans,* as compared to *Moraxella nonliquefaciens* Acta path. microbiol. scand. *59* 200–204 1963.
3 *Henrichsen J* Bacterial surface translocation a survey and a classification. Bact. Rev. *36* 478–503 1972.
4 *Henrichsen J.* On twitching motility and its mechanism. Acta path. microbiol. scand. Sect B, *83* 187–190 1985
5 *Henrichsen, J & Blom J* Correlation between twitching motility and possession of polar fimbriae in *Acinetobacter calcoaceticus* Acta path. microbiol. scand. Sect. B, *83* 103-115 1975.
6. *Hochberg M S & Folkman J* Mechanism of size limitation of bacterial colonies J. infect. Dis. *126* 629–635 1972.
7 *Kreutzer H J H* Protein concentration and dye uptake after agar gel electrophoresis. Clin chim. Acta *12* 575 578, 1965
8 *Lautrop H* *Bacterium anitratum* transferred to the genus *Cytophaga.* Int. Bull. bact. Nomencl. *11* 107–108, 1961
9 *Møller V.* A simplified apparatus for the measurement of the strength of agar Manuscript in preparation
10 *Piéchaud M* Mobilité chez les *Moraxella* Ann. Inst. Pasteur *104* 291–297 1963
11 *Stanier R Y* Studies on nonfruiting myxobacteria. I *Cytophaga johnsonae* n.sp. a chitin-decomposing myxobacterium. J Bact. *53* 297–315 1947

ON TWITCHING MOTILITY AND ITS MECHANISM

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Henrichsen, J On twitching motility and its mechanism. Acta path. microbiol. scand. Sect. B, 83 187–190 1975.

Previously published reports have established a correlation between twitching motility and the possession of polar fimbriae in all cases examined. Twitching motility was shown to be highly dependent on the availability of liquid at the agar surface. In the present paper experiments are reported that establish: 1) the dependence of twitching on the existence of a layer of liquid of a particular thickness, 2) the production of such a liquid layer surrounding areas of growing organisms, and 3) the affinity of twitching bacteria for the air-water interface. Reasoning from these facts, it is postulated that the demonstrated affinity for the air-water interface is conferred upon the cells by the polar fimbriae. It is also suggested how the movements might be generated.

Key words Twitching motility mechanism.

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"Twitching motility which may lead to the production of spreading zones on agar surfaces, has been defined as a kind of surface translocation produced by an unknown mechanism. The micromorphological pattern of the spreading zone is varying but not as organized as in swarming and gliding The cells move predominantly singly The movement appears as intermittent and jerky" (10) This kind of motility has been shown to occur in a variety of gram-negative bacterial species (12) and in *Streptococcus sanguis* (17) Polar fimbriae have been observed on all twitching strains examined in the electron microscope (14 15 16, 17) and reversely all strains with polar fimbriae have been shown to exhibit twitching motility—with the exception of a gliding strain (12) The conditions favourable for bringing this motility to light have been shown to include the use of only slightly dried dilute media and incubation in a humid atmosphere. The nature of the agar surface as well as the surface tension of the water phase of the medium was also found to be of importance (13) Finally it has been argued that the electrokinetic potential of twitching bacteria might be involved (13)

In this paper I report some simple experiments, the conclusions of which, considered together with the already existing body of information, have led me to postulate that polar fimbriae confer an affinity for air water interfaces upon the cells.

MATERIALS AND METHODS

Media inoculation incubation and agar plate microscopy were as described before (10)

The development of colonies of twitching Acinetobacter calcoaceticus *strains* was followed from the single cell stage by agar plate microscopy at hourly intervals of cultures at 30 C on cytophaga agar medium no. 62. For microscopy the plates were removed to room temperature, but returned to the incubator after the observation which did not exceed 5 minutes. The development of a particular colony could be followed by marking the bottom of the Petri-dish so that each time it could be placed in exactly the same position on the microscope stage by taking down the position of the coordinate indicators of the microscope stage with the colony in the centre of the field of vision.

RESULTS

1 *Twitching Motility in Microcolonies Requires a Minimum Number of Cells*

Twitching motility at its earliest began when the microcolony consisted of 50–100 cells. Many strains would not start to exhibit twitching until there were many more cells.

2. *Twitching Motility Requires a Surface Film of Liquid of a Minimum Thickness*

Cells exhibiting twitching were transferred by a platinum loop from the spreading zone on to the surface of a fresh preheated cytophaga agar plate. Motility of the transferred cells was not observed unless liquid was also added, *e g* by making a slight depression in the agar surface with the platinum loop or by applying a cover slip, one corner covering a drop of liquid previously placed on the plate. In this way areas with layers of fluid of different thickness could usually be created. If areas without added fluid were first examined under the microscope, followed by examination of areas where the thickness of the layer of fluid was gradually increasing, it was observed that the cells in the area without fluid did not move and subsequently gradually increasing degrees of twitching activity of the cells were seen as the thickness of the fluid layer increased until Brownian movements of some cells began to occur (*vide infra*) as an expression of abundant fluid.

3 *Surface Liquid Concentrates Around Bacterial Colonies*

In a strain that spreads by swimmin motility *e.g. Bacillus cereus* it is not all o the cells in the spreading zone that swim Th cells at the verge of the spreading zone are a rest (cf with Fig 8 in Ref 10) because th surface film of liquid outside the spreadin zone is too thin for swimming motility to tak place. For the same reason the movements o the cells are arrested at the verge of a spread ing zone produced by twitching motility

Swimming cells of *B. cereus* were tran ferred by a platinum loop to a spreading zo produced by a twitching strain of *Acinetobac ter calcoaceticus* Swimming of the *Bacill* cells was only evident in the most central pa of the spreading zone where the layer o surface fluid therefore must be thicker tha in the outer part of the spreading zon Swimming cells were also transferred to th periphery of a colony of *A calcoaceticus* on a medium that inhibits spreading due to twitch ing motility *e.g* cytophaga agar with 5 pe cent (w/v) tryptone or with agarose instea of Bacto agar (13) In this case the *Bacill* cells would swim *around* the colony indicat ing that surface liquid is concentrated aroun such colonies. This also shows that the laye of surface fluid required for swimming moti ity to take place must be thicker than tha required for twitching motility presumabl because bacteria swim within the liquid, whi twitching is exhibited by bacteria in the air water interface. The same conclusion may b reached if spreading zones of flagellate *Pseudomonas* strains with twitching motilit are studied Quite often cells were seen t swim, though abortively in the innermo part of the spreading zone, while at the sam time only twitching motility was displayed b the cells of the more peripheral parts of th spreading zone.

4 *Twitching Bacteria Display Affinity for the Air Water Interface*

If a drop of liquid was placed on top of t spreading zone of an *A calcoaceticus* strai

with twitching motility long cells from the spreading zone, known to possess polar fimbriae (14) could be seen to float on top of the drop exhibiting sudden side-ways jumps in the horizontal plane as well as turning around one pole in the vertical plane. These cells could be distinguished from those of the more central parts of the colony because they were much longer The shorter cells, known to be devoid of polar fimbriae, were found within the drop displaying Brownian movements.

5 *Only Growing Cells Display Twitching Motility*

Twitching motility could reversibly be brought to a halt by lowering the temperature to 4 C. Exposing anaerobic strains with twitching motility to atmospheric air resulted in loss of motility too. Formaldehyde vapour that killed the bacteria, irreversibly stopped twitching motility

DISCUSSION

In strains which possess the potential of performing twitching motility this motility can be observed when the polarly fimbriated—and growing—cells are situated on a region of an agar surface where the film of liquid has a thickness exceeding a certain critical size. It would therefore seem reasonable and in concordance with experimental facts to postulate that the polar fimbriae confer the demonstrated affinity for the air water interface upon the cells, *e.g* by being composed of a protein with a high concentration of hydrophobic amino acids in the surface If the polar fimbriae exhibit hydrophobic effects, the fimbriated bacteria should predictably adhere to hydrophobic material like glass surfaces. This has regularly been observed to be the case (*Henrichsen* unpublished observation) Lipoproteins may also display hydrophobic effects, but at present it appears to be most probable that the polar fimbriae are composed of protein because gonococcal fimbriae have been purified and claimed to

Gliding motility may also take place on the surface of a film of liquid (2)—in the air water interface (6)—and gliding bacteria most often show an affinity for air water interfaces (*Henrichsen* unpublished observation) Since gliding bacteria do not regularly possess fimbriae (15) this affinity must be due to cell surface properties.

Of course, suggestions about how twitching movements are generated in the air water interface can at present only be speculative, but one or more of the following five factors could be at work

1) Interfacial tension phenomena. *Mudd & Mudd* (19) in 1924 described jerky and spasmodic movements of otherwise non-motile bacteria in an interface with high interfacial tension.

2) Local changes in the bacterial surface charge density that presumably is in a dynamic state.

3) Affinity of the hydrophilic cells for the water phase due to their negatively charged groups.

4) Brownian movements of the cells confined to the interface

5) Long range electrostatic repulsive forces between the agar gel and the cells accordingly movements should be modified by factors that affect the electrokinetic potential of the cells and experiments on the effect of adding different ions in different concentrations to the substrate can perhaps be interpreted in support of the significance of this factor (13)

On purely speculative grounds it has been suggested that the fimbriae of bacteria with twitching motility might be able to contract or retract (7) and the resemblance of the filaments of *Pseudomonas testosteroni* to F actin filaments has been pointed out (8) Obviously this kind of contractility of polar fimbriae would offer a very good explanation of the generation of twitching motility were it not that it is very difficult to envisage how such type of contractile machinery could function outside the cell milieu proper In addition twitching motility is unaffected by

worth noticing in this connection that strong arguments in favour of rotation of bacterial flagella as rigid helices instead of propagation of helical waves along the flagellar filaments recently have been advanced (1 21)

A retraction model for the F fimbriae involved in conjugation (5 20) and for *Pseudomonas aeruginosa* RNA phage fimbriae (3) has been proposed. Contraction by means of dislocations has been proposed for the tail sheath of T-even bacteriophages (9) and for fimbriae of *Pseudomonas echinoides* (18) Common to these processes is, however that they are not repetitive they only occur once with the specific purpose of bringing cells together Therefore, it does not appear to be likely that such processes should partake in twitching motility

REFERENCES

1 *Berg, H C & Anderson R. A* Bacteria swim by rotating their flagellar filaments. Nature *245* 380–382, 1973

2. *Bisset K A* The cytology and life-history of bacteria. 2nd ed. Livingstone, Edinburgh and London 1970 p 39–40

3. *Bradley D E* Evidence for the retraction of *Pseudomonas aeruginosa* RNA phage pili. Biochem. Biophys. Res. Comm. *47* 142–149 1972.

4 *Buchanan T M Swanson J., Holmes K. K. Kraus S J & Gotschlich E. C* Quantitative determination of antibody to gonococcal pili. Changes in antibody levels with gonococcal infection. J clin. Invest. *52* 2896–2909 1973

5 *Curtiss III R.* Bacterial conjugation. Ann. Rev Microbiol. *23* 69–136, 1969

6. *D ews G & Nultsch W.* Spezielle Bewegungserscheinungen von Einzellern. In Ruhland W (Ed.) Handbuch der Pflanzenphysiologie. Vol 12/2. Springer Verlag Berlin-Göttingen-Heidelberg 1962, p 883.

7 Frøholm, L. O & Bøvre, K. The fimbriated state and competence in genetic transformation of three *Moraxella* species. In Archer L. J (Ed.) Bacterial transformation. Academic Press, London 1973 p. 125–139

8. *Fuerst J A & Hayward A. C* Surface appendages similar to fimbriae (pili) on *Pseudomonas* species. J gen. Microbiol. *58* 227–237 1969

9 *Harris W F & Scriven L. E.* Cylindrical crystals, contractile mechanisms of bacteriophages and the possible role of dislocations in contraction. J theor Biol. *27* 233–257 1970

10 *Henrichsen J.* Bacterial surface translocation a survey and a classification. Bact. Rev. *36* 478–503 1972

11 *Henrichsen J* Gliding and twitching motility of bacteria unaffected by cytochalasin B. Acta path. microbiol. scand. Sect. B, *80* 623–628, 1972.

12. *Henrichsen J.* The occurrence of twitching motility among gram-negative bacteria. Acta path. microbiol. scand. Sect. B, *83* 171–178, 1975

13. *Henrichsen J* The influence of changes in the environment on twitching motility Acta path. microbiol. scand. Sect. B, *83* 179–186, 1975

14 *Henrichsen J & Blom J:* Correlation between twitching motility and possession of polar fimbriae in *Acinetobacter calcoaceticus*. Acta path. microbiol. scand. Sect. B, *83*. 103–115 1975

15 *Henrichsen J & Blom J* Examination of fimbriation of some gram-negative rods with and without twitching and gliding motility Acta path. microbiol. scand. Sect. B, *83*. 161–170, 1975

16. *Henrichsen J Frøholm L. O & Bøvre K* Studies on bacterial surface translocation 1 Correlation of twitching motility and fimbriation in colony variants of *Moraxella nonliquefaciens M bovis* and *M kingii*. Acta path. microbiol. scand. Sect. B, *80* 445–452, 1972

17 *Henriksen S D & Henrichsen, J* Twitching motility and possession of polar fimbriae in spreading *Streptococcus sanguis* isolates from the human throat. Acta path. microbiol. scand. Sect. B, *83* 133–140 1975.

18. *Meyer F & Schmitt R* Elektronenmikroskopische, diffraktometrische und disc-elektrophoretische Untersuchungen an Fimbrien des sternbildenden Bodenbakteriums *Pseudomonas echinoides* und einer nicht-sternbildenden Mutante. Arch. Mikrobiol. 79 311 326, 1971

19 *Mudd S & Mudd E. B. H* The penetration of bacteria through capillary spaces. IV A kinetic mechanism in interfaces. J exp Med. *40* 633–645 1924

20 *Novotny C P & Fives Taylor P.:* Retraction of F pili. J Bact. *117* 1306–1311 1974

21 *Silverman M & Simon M* Flagellar rotation and the mechanism of bacterial motility Nature *249* 73–74 1974

Acta path. microbiol. scand. Sect. B, 83 191–200, 1975

A STUDY OF THE ULTRASTRUCTURE OF THE BUFFALO POX VIRUS

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Bloch, B. & Lal, S. M. A study of the ultrastructure of the buffalo pox virus. Acta path. microbiol. scand. Sect. B, 83 191–200 1975

Buffalo pox virus (strain BP 4) has been studied by electron microscopy of sections of infected chorioallantoic membrane cells. The ultrastructure of the virus has been described and compared with the ultrastructure of vaccinia virus. No differences between the two viruses were found. In relation to the ways of release two forms of mature virions have been described. These are probably analogous to the previously described C- and M-form of vaccinia.

Key words Buffalo pox virus ultrastructure.

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The buffalo pox virus was discovered a few years ago when *Singh & Singh* (1967) isolated a pox virus from an outbreak of buffalo pox in India.

Preliminary data were given by *Baxby & Hill* (1969) who examined four strains supplied by *Singh & Singh*. They stated that the virus was a member of the vaccinia subgroup of pox viruses and that it could be distinguished from other members of this group. Later *Kataria & Singh* (1970) showed that the virus was related to cow pox and even more closely to vaccinia. This conclusion has been further supported in recent works (*Baxby & Hill* 1971 *Hill et al.* 1972, *Lal & Singh* 1973 a and b) and the virus is now generally considered to be a separate member of the vaccinia subgroup of pox viruses. With a view to a further elucidation of the ultrastructure of the virus, an electron microscopical examination of chorioallantoic membrane (CAM) infected with the buffalo pox virus was carried out and the following report contains the results obtained.

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MATERIALS AND METHODS

Virus. Buffalo pox virus, strain BP 4 originally isolated from an outbreak in Hissar India, was received from professor *I P Singh* as infected rabbit skin preserved in 50 per cent glycerol saline. Virus was inoculated into the CAM of 12-day old chick embryos. The eggs were incubated at 37 C and the membranes were harvested at 24 36 and 48 h post infection.

Electron microscopy Pock-containing pieces of CAM, rinsed in saline, were cut out and fixed in 2.5 per cent glutaraldehyde in 0.1 M sodium phosphate or sodium cacodylate buffer (pH 7.3) for two hours at room temperature. The samples were postfixed overnight in 1 per cent ...

cording to *Kurtz* (1961) Sections were cut on an LKB ultratome I stained with uranyl acetate and lead citrate and examined with a JEM 100 B electron microscope.

Thick sections, 1–2 µm, were stained with 1 per cent toluidine blue in water and used for preliminary orientation within the tissue blocks.

RESULTS

At the border of a pock (36 hour after infection) virus was found in the majority of the CAM cells. The virus found was in size and ultrastructure similar to vaccinia virus.

The Mature Virion

The mature virion had the characteristic shape of the pox virus, traditionally characterized as brick-shaped. However seen transversally sectioned, the virions were of ellipsoidal shape (Fig 1) and sectioned longitudinally—in parallel to the flattest side—they had the shape of a trapezoid with rounded corners (Fig 2) The majority of the virions were 280–330 nm long and 200–250 nm wide. The central part of the virion was occupied by a core. In longitudinal sections it appeared trapezoidal or rectangular while in transversal sections it normally appeared dumbbell-shaped. The contents of the core were composed of a strongly osmiophilic fibrillar substance in an unstained matrix. The osmiophilic substance was preferably situated in the periphery of the core and as diffuse walls or protrusions into the interior of the core, partially dividing it into 2 or 3 compartments oriented in the longitudinal direction. Seen in sections parallel to the flat sides of the core, these walls could have the shape of an oblong S or C (Fig 1 and 2)

The core was lined by an app. 6 nm thick amorphous grey layer bounded by a somewhat more dense layer on which an app 8 nm thick layer—composed of rodlike subunits oriented perpendicular to the base—was situated (Fig 1 and 3) Between this layer and the outer unit membrane of the virion, an almost homogenous dark greyish layer was positioned. At the ends of the virion, this layer was app. 6 nm thick, while it along the flatter sides of the virion expanded to a thickness of 60 nm, forming the two lens-shaped bodies normally referred to as the lateral bodies. The contents of the lateral bodies appeared less dense than the linings which occasionally could have the appearance of a dense line (Fig 3) *Normally the limiting* unit membrane of the virion would be straight, but in some cases it had a wavy appearance, forming 15–20 nm broad profiles (Fig 2) The outer layer of the unit membrane was thicker and more heavily stained than the inner layer

The Developmental Bodies

In conformity with other pox viruses, the mature virions seem to be developed from the almost spherical bodies referred to as developmental bodies. These were found within or at the border of a well-defined area referred to as a virogenic matrix or B type inclusion in the cytoplasm of the infected cells (Fig 4)

The shells of the developmental bodies were seen to emerge as arcs or spheres, measuring app 320–350 nm in diameter They were composed of an 8 nm wide unit membrane on the outer convex side of which

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With the exception of the scale bar in Fig. 4, the scale bars in all other figures equal 0.2 µm

Fig 1 Mature virions lying in the cytoplasm The virion at "A" is cut *longitudinally* and perpendicular to its flat sides. The virion at "B" is cut transversally Note the two lightly stained areas in the heavily stained core Also the core lining membrane studded with spicules can be distinguished (arrow)

Fig 2 Virion sectioned parallel to the flat sides. The dark walls dividing the interior of the core can be seen. The outer layers of the virion are of an undulated appearance (arrow)

Fig 3 Virion sectioned as "A" in Fig. 1 The spicules of the core membrane can be seen (arrow head) An almost membraneous lining of one of the lateral bodies (L) can be seen (arrow) The virion is surrounded by the unit membrane of the inner release vacuole.

Fig 4 Part of an infected cell containing matrix (M) with developmental bodies (arrow) *Many* mature virions are scattered in the cytoplasm. Scale bar equals 1 µm.


A
B

2
3

M
M


5
6
7

10 nm long spicules were attached (Fig. 5) Usually the single spicules could not be distinguished due to superimposition, but at certain orientations and on certain abnormally formed, e.g. tubular virions (Fig 6) the spicules were distinguishable. Generally this appearance of the composit shell was observed along the entire length of the shell, but sometimes the membrane of an opened micelle was found to be attached to the unit membrane at the edge of such shell (Fig 8) Both within the matrix and outside it in the cytoplasm, regions of a dense flocculated substance—more osmophilic than the matrix material—were found from which material was seen to protrude into unclosed shells (Fig. 7) In one case, an accumulation of this dense substance was found in part to consist of a crystalline component arranged as a lattice of parallel, app 6.5 nm wide, lines at 10 nm intervals (Fig. 9)

Around the matrix, bundles of 25 nm wide tubules were commonly found (Fig. 10) Frequently 50 nm wide closed micelles were observed together with these tubules. Such micelles were usually positioned peripherally in the matrix (Fig. 8) In the cytoplasm of a number of cells—often near the matrix—some highly osmiophilic laminar structures were found. Most often they consisted of a pair or a triplet, of short parallel flat smooth cisternae devoid of ribosomes. The contents of these cisternae were heavily stained. Occasionally this complex was seen to be embedded in or surrounded by small amounts of material with the same density as the substance that was seen to have protuded into the interior of some of the shells (Fig 11)

Fig. 5. Part of developmental body the shell of which is composed of a unit membrane with spicules (arrow) The virion is of more than average size.

Fig. 6 Part of cell containing a tubular developmental body The spicules of the shell can be distinguished.

Fig 7 Accumulation of viroplasm (VP) bordered by three shells of developmental bodies. The complex is surrounded by cytoplasm with ribosomes and endoplasmic reticulum. Note the spicules on the lower shell (arrow)

Fig. 8 Piece of a shell of a developmental body A micelle with a unit membrane is attached to the shell unit membrane

Fig 9 Accumulation of viroplasm (VP) partly consisting of a crystalline component. A shell of a developmental body can be seen in the lower left corner

The Intermediate Virions

Together with those developmental bodies that were almost filled with a flocculated substance, other developmental bodies could be seen. In these the flocculated contents were partly replaced by a dense mass lying in an unstained space peripherally in the body (Fig. 12) At certain orientations, some of these dense masses could appear striated and show parallelly arranged, app 3 nm wide, threads lying at intervals of app 5 nm (Fig 13) More seldom, developmental bodies containing a more or less complete core were found in the matrix. The wall of these cores could be composed of an amorphous grey layer bounded by a somewhat denser layer (Fig 14) or of such a layer covered with fine spicules like the core wall of the mature virion (Fig 15) The developmental bodies containing a core were usually missing parts of the outer spicule layer of the shell which frequently was of a dentate aspect (Fig. 14) as if the body had shrunk. In developmental bodies, devoid of shell spicules but with a spicule covered core, also the lateral bodies could usually be recognized (Fig. 16) The developmental bodies at this state were normally found on the border of the matrix or just outside it.

The Release of the Virions

In the cytoplasm outside the matrix, only virions of the mature appearance were usually found. They were scattered in all internal parts of the cytoplasm. In most cells long, app. 10 nm wide bands of a cytoplasmatic material were observed to be attached to the surface of the virions until they were covered with a diffuse—sometimes beaded—

app 20 nm wide layer (Fig 17 and 21) This layer could in some cases be of an almost laminar appearance (Fig 18)

Coated virions were frequently present in the intercellular space in the vicinity of ruptured cells partly filled with coated virions.

Also uncoated virions were present in the cytoplasm of some cells. They were frequently lying in close contact with flattened cisternae (Fig. 19) or the single virions could be included in double walled vacuoles (Fig 20) the outer vacuole of which sometimes contained two or more virions separately included in internal vacuoles. Virions in separate vacuoles were often observed to be released from the plasma membrane of the cells leaving an indentation in the otherwise intact plasma membrane (Fig 21) Outside the cells, both coated and uncoated virions were found together with virions included in intact or ruptured vacuoles.

DISCUSSION

The object of this study has been to determine whether or not the buffalo pox virus ultrastructually could be distinguished from the closely related pox viruses. As vaccinia virus is the prototype of the viruses of the variola vaccinia subgroup the buffalo pox virus will be compared with this virus.

It has not been attempted to examine the complete infection cycle and therefore no samples from early infections have been included in this material. For that reason, mainly later developmental events are described.

The virogenic matrix in which the developmental virions emerge is typical of several other viruses of the pox group and in most cases quite similar to that of vaccinia, described by *Dales & Siminovitch* (1961) and *Dales* (1963) The regions of the dense flocculated substance that was seen to protrude into unclosed shells are similar to the foci of viroplasm in the cytoplasm of L cells infected with vaccinia virus (*Dales* 1963) The nature of the crystalline lattice in Fig 9 seen to be lying in such a region of viroplasm, is at present unknown.

The micelles found near or inside some matrices are very similar to those observed by *Tripier et al.* (1973) who described them in connection with vaccinia infected arginine-deprived KB cells. The micelles and perhaps the tubules sometimes found at the border of matrices may be considered to be a structural aspect of late pox infections. As regards the appearance and dimensions of the shells of the developmental bodies, they are similar to the shells of vaccinia described by *Dales & Mosbach* (1968) The rare occurrence of shells covered with well-distinguishable spicules could be explained by the assumption that these spicules are only observable at certain orientations that are most easily found in connection with some erratic shell forms.

In conformity with many other pox viruses, the morphogenesis of the core seems to begin with the formation of an osmophilic body often referred to as a nucleoid. Some of the

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Fig 10 Bundle of tubuli. Such tubuli are also bordering the outside of the matrix with developmental bodies.

Fig 11 Groups of flat parallel cisternae associated with viroplasm. The contents of the cisternae are heavily stained.

Fig 1 Matrix containing developmental bodies. The contents of some of these form dense mass placed near the shell. Within matrix (M) a cisterna of the rough endoplasmatic reticulum (ER) is present.

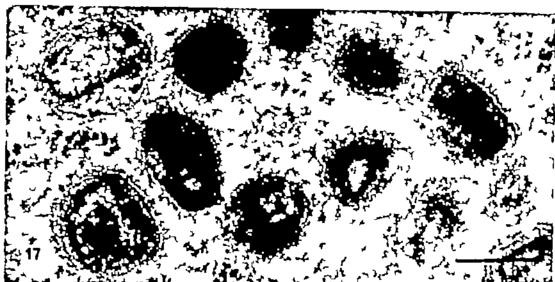
Fig 13 Condensed mass in a developmental body. The mass seems to be composed of almost parallel threads.

Fig 14 Intermediate virion placed in the matrix. The lower amorphous part of the core membrane has been formed (arrow head) The spicule layer of the shell is missing at the arrows.

Fig 15 Intermediate virion partly surrounded by cytoplasm with ribosomes. The core membrane is covered with spicules (arrow)

Fig 16. Intermediate virion placed in the cytoplasm together with mature but uncoated lines. The core membrane is complete and material has been deposited at the site of the lateral bodies. The outer shell has partly lost its spicules.


10
11
M
ER
12
13
14
15
16


17


18


19


20


21

served nucleoides show a striation which is milar to striation patterns which have been oted in the case of vaccinia (*Dales* 1963) nd some other pox viruses.

As mentioned before, the early intermediate forms of the virus are only rarely found. his is probably due to a very short transition me. However some observations (Fig 14) ndicate that the developing core initially is ned by an amorphous grey layer bounded by somewhat more dense layer on which the picules later attach. The final core wall is imilar to the core wall of the mature vaccinia virion (*Easterbrook* 1966)

Late intermediate forms of the virions, containing the emerging lateral bodies and a picule covered core, are more frequently ound, also at locations within the cell where he condensed mature virions normally are resent. This could indicate that the normal hrinkage of the virion can be prevented, possibly because the uncondensed virions erratically are covered with an additional coating before they have reached the condensation or shrinkage phase. However the great majority of the virions seems to obtain the condensed appearance before they became coated.

Fig 17 Mature virions in the cytoplasm. The majority of virions are more or less coated with undulating membranes. Also the less condensed virion in the upper left corner is coated to some extent.

Fig. 18 Mature virion with heavy coat which partly is of flocculated appearance and partly (arrows) of a membraneous character. This could indicate that this flocculated coat can participate in the formation of the release vacuole.

Fig 19 Uncoated and incompletely condensed virion to which flattened cisternae are adhering. This could represent an early stage in the formation of the double release vacuole

Fig 20 Mature virion in double release vacuole near the surface of the cell. Note that the surface of the virion is uncoated.

Fig 21 A mature virion included in an inner release vacuole which seems to be expelled as a result of fusion of the outer release vacuole with the cell surface.

Apparently the virions can be released from the cells in two different ways. The plasma membrane of cells heavily loaded with mature and coated virions may after rupture and the virions be released, still wearing the surface coating. Alternatively a virion can leave a cell after being included in a double walled release vacuole. In this case, the outer membrane of the release vacuole probably fuses with the plasma membrane and the virion included in the inner vacuole will be released, leaving an intact plasma membrane behind. Occasionally this inner vacuole still in contact with the cell surface ruptures, as also virions included in the inner membrane are found in the intercellular space. While the virions released in vacuoles possibly could represent a form of the virus analogous with the C-form of vaccinia described by *Westwood et al.* (1964) the coated virions could represent an equivalent to the vaccinia M form also described by these authors. The M-form has been extensively examined by negative staining but has not been identified in sections. However an outer coat of antigen has been demonstrated by *Morgan et al* (1962) using ferritin-conjugated antibodies.

Although some observations in the present study may indicate that the release vacuoles of buffalo pox virus are derived directly from the endoplasmatic cisternae (Fig. 19) other observations may indicate that the surface coating itself is transformed into laminar structures able to form the membranes of at least the inner release vacuole (Fig. 18) Such a transformation of the coating into membranes could explain the fact that the M form of vaccinia virus can be converted into the C-form or an intermediate form in different ways, as described by *Westwood et al.* (1964)

The present investigation has not revealed any ultrastructural difference between buffalo pox virus and vaccinia virus. This is in good accordance with the serological findings reported by *Lal & Singh* (1973 b) Their observations as well as the present ultrastructural observations support the inclusion of buf

falo pox virus in subgroup I (the variola vaccinia subgroup) of the poxviruses.

The authors are indebted to professor *I P Singh* for supplying the buffalo pox virus. The technical assistance of Mrs. *I Szabo* is highly appreciated.

REFERENCES

Baxby D & Hill B J., Buffalopox virus. Vet. Rec. *85* 315–316 1969

Baxby D & Hill, B J., Characteristics of a new poxvirus isolated from India buffaloes. Arch. ges. Virusforsch. *35:* 70–79 1971

Dales S: The uptake and development of vaccinia virus in strain L cells followed with labelled viral deoxyribonucleic acid. J Cell Biol. *18* 51–72 1963

Dales S & Mosbach E. H Vaccinia as a model for membrane biogenesis. Virology *35* 564–583 1968.

Dales S & Siminovit h L. The development of vaccinia virus in Earle s L strain cells as examined by electron microscopy J Biophys. Biochem. Cyt. *10* 475–503 1961

Easterbrook K B Controlled degradation of vaccinia irions *in vitro:* An electron microscopic study J Ultrastruct. Res *14* 484–496, 1966.

Hill, B J., Baxby D & Douglas H W Micro-electrophoresis of enzyme and chemically treated viruses and cores of vaccinia, buffalopox, white and alastrim. J gen. Virol. *16* 39–46, 1972.

Kataria R. S & Singh I P Serological relationship of buffalopox virus to vaccinia and cowpox viruses. Acta virol., (Prague) *14* 307 311, 1970.

Kurtz S M A new method for embedding tissues in Vestopal-W J Ultrastruct. Res. *5* 468-469 1961

Lal S M & Singh, I P., Buffalopox virus. Arch ges. Virusforsch. *40* 390–391 1973 a.

Lal S M & Singh I P Serological characterization of buffalopox virus. Arch. ges. Virusforsch. *43* 393–395 1973 b.

Morgan C Rifkind R A & Rose H M: The use of ferritin-conjugated antibodies in electron microscopic studies of influenza and vaccinia viruses. Cold Spring Harbor Symp. Quant. Biol *27* 57–65 1962.

Singh, I P & Singh S B. Isolation and characterization of the aetiologic agent of buffalopox J Res., (Ludhiana) *4* 440-449 1967

Tripier F Obert G. & Kirn A., Étude ultrastructurale de l'effet de la carence en arginine sur la morphogenese du virus vaccinal J. Ultrastruct. Res. *42* 121–135 1973

Westwood J C N Harris W J., Zwartouw H T Titmuss D H J & Appleyard G. Studies on the structure of vaccinia virus. J gen. Microbiol. *34* 67–78, 1964

Acta path. microbiol. scand. Sect. B, 83 201–210, 1975

INFECTION OF *MICROTUS ARVALIS* (COMMON VOLE) WITH *MYCOBACTERIUM TUBERCULOSIS* AND *MYCOBACTERIUM BOVIS*

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Jespersen, A. Infection of *Microtus arvalis* (common vole) with *Mycobacterium tuberculosis* and *Mycobacterium bovis*. Acta path. microbiol. scand. Sect. B, 83 201–210 1975

The aim of the study has been to ascertain whether *Microtus arvalis* (common vole) is strongly susceptible to *M. bovis* but resistant to *M. tuberculosis* as is the case with other members of the vole family or whether it is susceptible to both species as demonstrated by *Robert Koch*. Groups of common voles have been injected subcutaneously or intraperitoneally with varying doses of finely dispersed suspensions of a virulent strain of *M. bovis* or *M. tuberculosis*. *M. bovis* multiplies strongly in the vole organism and a dose as small as 8 viable units provokes a progressive infection with rapid fatal outcome. Autopsy shows considerable processes at the site of injection and in the lymph glands. Tubercles are observed quite frequently in the lungs, but seldom in the liver spleen and kidneys. The organs contain a large number of tubercle bacilli, the caseous lymph glands enormous numbers. In contrast, the virulence of *M. tuberculosis* is low. None of the doses used, the highest being 6×10^{8} viable units, provokes progressive infection in the animals injected subcutaneously and only in a few of those injected intraperitoneally. The macroscopical findings are inconsiderable and it is characteristic of the *M. tuberculosis* infection that the lymph glands are seldom enlarged and have become caseous in exceptional cases only. The number of bacteria in the organs is small, except in the few animals in which the infection becomes progressive. The conclusion drawn from the experiment is that *Microtus arvalis* is susceptible to *M. bovis* but strongly resistant to *M. tuberculosis* and in this respect resembles other members of the vole family examined hitherto.

Key words: *Mycobacterium tuberculosis*; *Mycobacterium bovis*; infection; *Microtus arvalis*.

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Robert Koch (1912) found that *Microtus arvalis** was far more susceptible than white mice to tubercle bacilli isolated from both man and cattle. His interesting and important experiments, performed already in 1884, have never been reproduced. *Microtus arvalis* belongs to the vole family and is distinct in appearance and mode of living from the murids (house mouse, white mouse, wood mouse, rat, etc.). Reports available from recent years show that voles are very susceptible to *M.*

* Called by Koch *Arvicola arvalis*, a name previously used for the species.

bovis but resistant to *M tuberculosis* This applies to *Microtus agrestis* (field vole) (*Wells* 1938 *Griffith* 1937 1939 1941) *Clethrionomys glareolus* (red mouse) (*Jespersen* 1954 *Hauduroy & Bize* 1957 *Bize* 1961) and *Arvicola terrestris* (vole rat) (*Jespersen* 1974) The present study was performed in order to ascertain whether there is difference in the susceptibility of *Microtus arvalis* to *M tuberculosis* and *M bovis* or whether as reported by *Koch* the susceptibility to both species is the same.

MATERIALS AND METHODS

Experimental 2 × 5 groups of 10 common voles were injected subcutaneously with dilutions 10^0 10^{-2} 10 10^{-6} and 10^{-8} of a strain of *M tuberculosis* or a strain of *M bovis* grown on Dubos fluid Tween medium and dispersed by means of ultrasonics. 2 × 5 groups of 20 common voles were injected intraperitoneally with the same bacterial suspensions of both strains. The animals were allowed to live until they died spontaneously the experiment being concluded 1 year after the inoculation. Smears from liver spleen, kidneys, lungs, lymph glands and possible abscess were made at autopsy In animals where no macroscopically visible tuberculous processes were found, or where these were small, culture was made from liver, spleen, lungs and lymph glands.

Experimental animals The common voles were bred at the farm belonging to Statens Seruminstitut and were 2 to 5 months old at the start of the experiment. They were distributed at random in glass cages with two animals per cage. Each experimental group consisted of equal numbers of males and females.

Bacterial strains Before the start of the experiment both *M bovis* strain E 6884B and *M tuberculosis* strain E 10883H were inoculated intraperitoneally into a guinea pig which was allowed to die spontaneously A suitable organ was used for culture on Löwenstein-Jensen medium and subculture was made on Dubos fluid medium with Tween.

Bacterial suspensions for infection About 10 ml of an 8-day-old culture of each strain was shaken until homogenous macroscopically and then exposed to ultrasonic treatment for 10 minutes at an intensity of 0.56 watt/cm² After treatment, the two suspensions contained about 50 per cent single bacteria, about 40 per cent units of two or three bacteria, about 10 per cent units of four to six bacteria, and no units of more than six bacteria. Suitable dilutions of the suspensions were inoculated on Löwenstein-Jensen medium and Dubos oleic acid albumin agar plates. The colony counts showed that each animal injected with 0.2 ml of dilution 10^{-8} had been given six viable units of *M tuberculosis* and eight viable units of *M bovis*

TABLE 1 *Virulence Determination of* M. bovis *Strain E 6884B and* M. tuberculosis *Strain E 10883H on Guinea Pigs and Rabbits*

Animal	*M bovis*			*M tuberculosis*		
	Viable units	Survival time (days)	Degree of tuberculosis*	Viable units	Survival time (days)	Degree of tuberculosis
Guinea pig	8 i p.	† 96	V	6 i.p.	† 92	V
	8 i.p.	† 112	V	6 i.p.	† 102	V
	8 i.p.	† 135	V	6 i.p.	† 109	V
	8 i p.	† 141	V	6 i p.	† 128	V
	8 p	† 156	V	6 i.p	† 128	V
	8 i p.	† 158	V	6 i.p.	† 143	V
	8 i.p.	† 166	V	6 i.p.	† 143	V
	8 i.p.	† 173	V	6 i.p.	† 150	V
	8 p.	† 173	V	6 i.p	† 162	V
	8 i p.	k 375‡	O	6 i p.	† 219	V
Rabbit	8.3×10^4 i.	† 21		6.0×10^4 i.v	k 121	O
	8.3×10^4 i.v	† 26		6.0×10 i.v	k 121	II

k = killed, † = died spontaneously *see Table 2, underlined figures = median survival time.
‡ Omitted from calculation of median survival time.

TABLE 2. *Subcutaneous Infection of Common Vole* (*Microtus arvalis*) *with M. bovis*

Dose and number of viable units														
10[illegible] 8.3 × 10[illegible]			10[illegible] 8.3 × 10[illegible]			10[illegible] 8.3 × 10[illegible]			10[illegible] 8.3 × 10			10[illegible] 8		
Surv. time (days)	Degree of tub.	Bacterial index	Surv time (days)	Degree of tub.	Bacterial Index	Surv. time (days)	Degree of tub.	Bacterial Index	Surv time (days)	Degree of tub.	Bacterial Index	Surv time (days)	Degree of tub.	Bacterial Index
34	IV	3.1	57	V	4.5	5‡	0	0	50	III	1.0	49	III	1.4
53	V	4.6	60	V	4.6	25	III	1.0	55	IV	3.4	65	III	2.2
54	V	4.4	60	IV	3.0	55	IV	2.4	70	IV	2.5	68	III	2.2
59	V	3.9	61	V	4.4	56	III	2.5	71	III	1.8	79	V	4.4
59	V	3.6	64	V	4.0	75	III	1.8	77	IV	2.9	86	V	4.8
59	IV	3.8	66	III	1.2	77	IV	2.7	82	V	4.0	95	IV	2.4
61	IV	2.6	78	V	4.5	77	V	3.2	86	IV	2.9	95	V	4.4
61	III	2.8	81	IV	3.6	95	V	4.8	95	V	4.8	112	V	3.6
75	IV	3.6	82	IV	3.4	99	III	2.6	109	V	3.2	116	V	3.2
89	V	3.2	84	III	2.8	100	V	3.6	111	V	3.2	129	V	4.4
Mean bact. index		3.3			3.6			2.5			3.0			3.3

‡ Omitted from calculation of median survival time and mean bacterial index.
Underlined figures = median survival time.

Degree of tuberculosis

Tub. 0 Organs normal, culture negative. Tub. I: No definite specific changes, culture positive. Tub II Small tuberculous changes of mainly regressive nature. Tub. III: Slight progressive tuberculosis. Tub. IV Medium progressive tuberculosis. Tub. V Severe progressive tuberculosis.

Bacterial index

0: No bacilli. 1: Culture positive. 2: 1–20 bacilli by microscopy 3 >20 bacilli to 1–2 bacilli per sight field. 4: >1–2 bacilli per sight field to 10–20 bacilli per sight field. 5: Innumerable bacilli per sight field.

The number of bacteria in liver spleen, kidneys, lungs and lymph glands was indicated by figures 0–5 The index for the individual animal is the average of these five figures.

TABLE 3 *Subcutaneous Infection of Common Vole* (Microtus arvalis) *with* M. tuberculosis

Dose and number of viable units														
10^{0} 6×10^{4}			10^{-1} 6×10^{3}			10^{-2} 6×10^{2}			10^{-3} 6×10			10^{-4} 6		
Surv time (days)	Degree of tub.	Bacterial index	Surv time (days)	Degree of tub.	Bacterial index	Surv time (days)	Degree of tub.	Bacterial index	Surv time (days)	Degree of tub.	Bacterial index	Surv time (days)	Degree of tub.	Bacterial index
53	I	0.3	90	I	0.3	22	I	0.3	88	I	0.8	83	0	0
93	I	0.3	127	I	0.3	39	I	0.3	88	0	0	150	II	0.3
117	I	0.5	179	II	0.4	172	0	0	183	0	0	202	I	0.3
176	II	0.7	183	0	0	183	I	0.8	185	I	0.3	290	0	0
258	II	0.3	209	0	0	208	0	0	189	0	0	339	I	0.7
267	I	0.5	218	0	0	239	I	0.7	217	I	0.3	k 375	II	0.7
371	II	0.7	237	0	0	291	I	0.5	227	0	0	k 375	III	1.2
k 375	III	0.8	299	I	0.3	358	0	0	263	0	0	k 375	0	0
k 375	II	1.3	354	I	0.5	k 375	II	1.0	300	0	0	k 375	III	1.0
k 375	III	1.0	Died before inf.			Died before inf.			355	0	0	k 375	II	1.0
Mean bact. index		0.6			0.2			0.4			0.1			0.5

k = killed.
Underlined figures = median survival time

Virulence determination of strains The same suspensions as those injected into the voles were used for virulence determination on guinea pigs and rabbits. The survival times given in Table 1 show that *M tuberculosis* is at any rate not less virulent for guinea pig than *M bovis*.

RESULTS

Subcutaneous Infection

M bovis (Table 2) Doses from 8.3×10^{4} to 8 viable units provoked progressive fatal tuberculosis in all animals. The survival times were prolonged the lower the dose. The mean survival times for the five groups were) 65 77 80 and 91 days, respectively

The autopsy findings were, on the whole, independent of dosage. At the site of injection there was a pea-sized abscess which had often emptied some of its content by perforating the skin. The subcutaneous lymph gland, which was affected in about half of the animals, was hempseed to pea-sized. The lumbar gland was systematically enlarged to the size of a date kernel, and both the lumbar and subcutaneous glands were always caseous. The mesenteric, portal and tracheal glands were moderately enlarged in about 40 per cent of the animals, mainly in those that survived for the longest period of time. There were no macroscopically visible tubercles in liver and kidneys. The spleen was generally enlarged, sometimes 5 to 10 times the normal volume, but, except in a single case, contained no tubercles. Tubercles in the lungs were found in 30 per cent of the voles and occurred the more frequently the longer the animals survived. In the 10^{-4} group tubercles were seen in six animals which died in the period 79th to 130th day after infection.

The numbers of tubercle bacilli in the organs were large and almost the same in all groups. The mean bacterial index in the five groups was 3.5 3.6, 2 5 3 0 and 3 3 respectively in order of decreasing dosage. The greatest numbers of bacilli were found in the caseous abscess and in the caseous lymph glands However also liver spleen and kidneys contained many bacilli despite the fact that no tubercles were observed in these organs. In the lungs, the number of bacteria was particularly great in the cases where tubercles had developed.

M tuberculosis (Table 3) No deaths attributable to tuberculosis occurred during the experimental period of 375 days. The survival times in the individual groups were not affected by the number of bacilli injected.

The autopsy findings were minimal. There was no development of abscess at the site of injection, except in four animals injected with the two largest doses, where small remnants of subcutaneous or subfascial abscesses were seen. In 15 voles (31 per cent) the regional lymph glands, *i.e* the subcutaneous or lumbar glands, were from pinhead to millet seed in size, but these were caseous only in two animals which were killed on the 375th day In seven voles (15 per cent) the mesenteric or portal glands were slightly enlarged but not caseous, and the tracheal glands were normal in all animals. The liver spleen and kidneys contained no tubercles, but in 11 animals (23 per cent) the spleen was enlarged to twice its normal volume, mainly in those surviving for the longest period of time. There were tubercles in the lungs of two animals killed on the 375th day the one infected with undiluted culture and the other with dilution 10^{-4}

The number of tubercle bacilli in the organs was small. Microscopy of smears revealed tubercle bacilli in the remnants of the abscesses and in the enlarged lymph glands. However in liver spleen and lungs, bacteria could only be demonstrated by culture. This was the case in about half of the animals in the remainder culture was negative. Tubercle bacilli were found more frequently in the lungs than in liver and spleen. The mean bacterial index for the five groups was 0 6, 0.2, 0 4 0 1 and 0.5 respectively in order of decreasing dosage. The index was highest for the voles killed on the 375th day

Intraperitoneal Infection

M bovis (Table 4) Doses from 8.3×10^{4} to 8 viable units provoked fatal tuberculosis.

TABLE 4 *Intraperitoneal Infection of Common Vole (Microtus arvalis) with M. bovis*

Dose and number of viable units														
10^{0} 8.3 × 10^{4}			10^{-1} 8.3 × 10^{3}			10^{-2} 8.3 × 10^{2}			10^{-3} 8.3 × 10			10^{-4} 8		
Surv time (days)	Degree of tub.	Bacterial index	Surv time (days)	Degree of tub.	Bacterial index	Surv time (days)	Degree of tub.	Bacterial index	Surv time (days)	Degree of tub.	Bacterial index	Surv time (days)	Degree of tub.	Bacterial index
14	V	3.8	32	V	5.0	7§	II	0.5	49	III	1.5	4§	0	0
19	IV	2.8	33	III	2.6	33	III	2.1	56	IV	3.0	54	V	4.6
23	V	4.4	49	V	5.0	45	I	0.7	57	III	1.6	56	III	1.7
24	V	5.0	51	IV	3.8	46	IV	3.4	60	III	2.6	67	IV	2.0
24	V	5.0	51	V	4.2	47	III	2.6	61	IV	3.1	75	V	4.2
24	V	5.0	52	V	4.0	54	V	4.6	67	V	4.6	76	V	4.4
25	V	4.8	52	V	4.2	57	V	4.2	70	V	4.4	81	V	4.6
25	V	5.0	54	V	3.8	67	V	3.5	70	IV	2.4	83	IV	2.5
25	V	4.8	55	V	4.6	73	V	4.2	70	V	4.2	86	V	5.0
26	V	5.0	56	V	4.6	74	V	4.8	76	IV	4.0	87	V	4.2
26	V	4.8	57	V	4.2	75	V	4.8	82	III	2.3	91	V	4.2
27	V	4.8	67	IV	3.3	76	IV	3.6	82	V	3.6	97	V	5.0
28	V	5.0	68	IV	2.6	81	V	3.6	85	V	5.0	101	IV	3.2
31	V	4.8	76	V	4.6	88	V	4.4	90	V	4.0	102	IV	3.2
31	V	4.6	79	V	5.0	88	V	4.2	91	V	2.6	108	IV	2.6
34	V	5.0	80	IV	3.0	90	V	3.2	102	V	3.6	113	IV	3.0
34	V	4.2	86	V	5.0	92	III	2.2	102	IV	2.6	123	V	3.4
40	IV	2.8	87	V	4.8	104	V	3.2	111	IV	3.2	127	V	4.8
55	IV	3.2	91	V	4.4	120	V	4.2	116	V	3.8	244§	0	0
67	V	4.6	106	V	5.0	147	IV	2.0	301§	0	0	341§	0	0
Mean bact. index		4.5			4.1			3.4			3.3			3.7

§ Omitted from calculation of median survival time and mean bacterial index.
Underlined figures = median survival time

TABLE 5. *Intraperitoneal Infection of Common Vole* (Microtus arvalis) *with* M. tuberculosis

						Dose and number of viable units								
10^{0} 6×10^{6}			10^{-2} 6×10^{4}			10^{-4} 6×10^{2}			10^{-5} 6×10			10^{-6} 6		
Surv time (days)	Degree of t b	Bacterial index	Surv time (days)	Degree of tub.	Bacterial index	Surv time (days)	Degree of tub.	Bacterial index	Surv time (days)	Degree of tub.	Bacterial index	Surv time (days)	Degree of tub.	Bacterial index
63	I	0 7	151	I	0.7	20	II	1 0	32	III	1.8	5‡	0	0
64	0	0	153	0	0	52	0	0	49	0	0	61	I	0.3
68	III	0 8	159	0	0	53	0	0	62	0	0	70	0	0
72	V	3.3	196	II	1.0	148	I	0.7	108	I	0.3	148	0	0
73	I	0.4	205	0	0	158	0	0	108	0	0	227	II	0 7
76	I	0.4	206	I	0.3	179	I	0.3	118	0	0	233	0	0
77	I	0.3	236	0	0	180	I	0.3	158	0	0	248	0	0
81	III	2.3	249	0	0	217	0	0	172	0	0	249	0	0
85	I	0.7	254	0	0	237	0	0	184	0	0	257	0	0
86	III	2.0	261	I	0.5	277	0	0	203	II	0.3	282	I	0.3
86	III	1.8	264	II	0.3	318	0	0	206	0	0	285	0	0
91	III	1.3	288	0	0	341	0	0	212	0	0	300	I	0.3
95	II	1.3	298	I	0.5	343	I	0.3	261	0	0	320	I	0.3
104	I	0.5	305	V	1.2	347	0	0	272	0	0	324	V	4 7
122	IV	2.2	357	I	0.7	368	I	0.3	272	I	0.3	333	I	1.0
150	I	0.3	364	I	0.3	k 376	II	1.0	298	0	0	335	II	1.3
185	III	1.0	k 376	II	1.0	k 376	II	0.7	312	0	0	352	IV	2.7
202	III	1.3	k 376	0	0	k 376	0	0	313	0	0	355	0	0
211	III	1 7	k 376	II	0.7	k 376	II	1.0	315	I	0.3	k 376	0	0
274	I	0.7	k 376	II	1.0	k 376	II	1.0	k 376	I	0.3	k 376	0	0
Mean bact. index		1.2			0.4			0.3			0.2			0.6

‡ Omitted from calculation of median survival time and mean bacterial index. k = killed.
Underlined figures = median survival time.

The median survival times in the five groups were 26, 57 75 76 and 87 days, respectively in order of decreasing dosage. In the voles injected with the undiluted suspension, the course was more rapid than in the subcutaneously injected animals (median survival time 26 versus 59 days) but in the groups 10^{-} to 10^{-4} the route of injection had no influence on the survival time. No sign of tuberculosis could be found in one vole in the 10^{-8} group (died on 301st day) and in two in the 10^{-6} group (died on 224th and 341st day respectively)

The autopsy findings were, on the whole independent of dosage, though they were slightly more advanced in animals surviving for the longest period of time infected with small doses. The omentum was almost constantly thickened and consisted of a uniform, yellow mass. As in the subcutaneously infected animals, the lymph glands were greatly enlarged and caseous. The portal, mesenteric and lumbar glands were generally the size of millet to hempseed, sometimes pea-sized. In the animals infected with small doses, a large number of hempseed to pea sized yellow glands were often found in the abdomen, situated singly or forming a conglomeration of coalescent glands. The spleen was constantly enlarged to 5 to 15 times the normal volume, and in eight animals (8 per cent) pinhead sized yellow tubercles had developed. There were no tubercles in liver and kidneys. Tubercles were found in the lungs of 27 animals (27 per cent) seven of these were infected with the smallest dose.

The number of bacilli in the organs was great, not only in the caseous lymph glands, but also in liver spleen, kidneys and lungs. The mean bacterial index was 4.5 4 1 3 4 3.3 and 3 7 respectively in order of decreasing dosage.

M tuberculosis (Table 5) The virulence of *M tuberculosis* was higher after intraperitoneal than after subcutaneous injection. The largest dose provoked chronic progressive tuberculosis, at any rate in some of the animals. The median survival time of the animals in that group was 86 days. Lower doses had no effect on the median survival time.

The autopsy findings were minimal. In half of the voles in the 10^{0} group, the lymph glands were up to millet-sized but not caseous. The spleen was 2 to 10 times enlarged in six animals. There were no tubercles in liver spleen, kidneys or lungs. Caseous tubercles were found in the lungs of one vole in the 10^{-2} group and in two voles in the 10 group they died on the 305th, 324th and 352nd day respectively The remaining animals in the groups 10^{-4} to 10^{-4} showed no definite signs of tuberculosis.

The number of bacteria in the organs was greatest in the 10^{0} group. Tubercle bacilli could be demonstrated by microscopy of smears from liver spleen, kidneys or lungs in 12 animals, and in six of the remaining animals by culture. The mean bacterial index was 1.2. In the groups 10^{-} to 10^{-6} the number of bacteria was clearly less, the mean index for the four groups being 0.4 0.3, 0.2 and 0.5 respectively The number of bacilli was greatest in the voles that survived for 300 days or more. As regards the above-mentioned three animals with tubercles in the lungs, the high number of bacilli in the organs—and particularly in the lungs—left no doubt that tuberculosis was the cause of death.

DISCUSSION

The author's experiments have shown that *Microtus arvalis* is highly susceptible to *M bovis* Doses from 8.3 × 10^{6} to 8 viable units provoked fatal infection in all animals injected subcutaneously and in all those injected intraperitoneally with the exception of three The reason for the absence of infection in these animals, injected with 83 or 8 viable units, is doubtless that the bacteria were injected into the intestinal canal instead of into the peritoneal cavity

It was characteristic of the *M bovis* infection that considerable macroscopically visible processes developed at the site of injection and in the omentum and lymph glands, while the processes in liver spleen, kidneys and

gs were less prominent. All organs contained large numbers of tubercle bacilli.

The virulence of *M. bovis* was influenced slightly by the route of injection, the median survival time being shorter in animals injected intraperitoneally with the undiluted suspension than in those injected subcutaneously. Using lower doses, the route of injection had no effect on the course.

The course of the *M. bovis* infection was more rapid in voles than in guinea pigs. The median survival time of voles injected intraperitoneally with 8 viable units was 87 days against 156 in guinea pigs injected by the same route with the same dose. The difference in weights of the animal species cannot be the sole explanation of the difference in survival times.

Microtus arvalis is strongly resistant to *M. tuberculosis*. A progressive infection was provoked in some of the animals injected intraperitoneally with 6×10^6 viable units, but only in very few of those given lower doses. No deaths attributable to tuberculosis occurred among the animals injected subcutaneously—not even in the group given 6×10^6 viable units. The autopsy findings were considerable and the number of tubercle bacilli in the organs small. However, the largest numbers of bacilli were found in the latter part of the experimental period, which would indicate that tuberculosis would have been a more frequent cause of death had all the animals been allowed to live until death occurred spontaneously.

In consequence of the great difference in susceptibility to the two species, *Microtus arvalis* can be used for species determination. In order to avoid the development of abscess at the site of injection and the evacuation of strongly infectious material, the intraperitoneal or intravenous method of injection would be preferable.

In contrast to the above-mentioned results, *Robert Koch* (1912) found that the common vole (*Arvicola arvalis*) was strongly susceptible to both *M. tuberculosis* and *M. bovis*. In his sixth experiment with pure cultures, *Koch* examined the virulence of six different strains isolated from man (lupus, joint tuberculosis, lymph gland tuberculosis, miliary tuberculosis and pulmonary tuberculosis) or from a case of cattle tuberculosis. Each strain was injected subcutaneously into four voles. Shortly after infection the inguinal glands could be palpated, and later the animals lost weight, had respiratory difficulty and died after 4 to 6 weeks. Autopsy revealed tuberculous processes in lymph glands, liver, spleen and lungs. The pathological processes were identical in all animals, both as regards the macroscopical appearance of the individual tubercles, their histological structure and content of tubercle bacilli. In his fourth experiment, *Koch* injected four voles with culture from a tuberculous lung of a monkey. The voles were killed after 53 days. At autopsy the inguinal glands were found to be greatly enlarged and caseous and both lungs, liver and spleen contained tubercles. The organs of five white mice injected with the same strain were apparently normal, except for one which had tubercles in the lungs. In the seventh experiment also, in which *Koch* infected 24 voles with culture from a tuberculous lung, the course was as described above, and all the animals died of generalized tuberculosis.

It is not possible to give a reasonable explanation of the discrepancy between *Koch*'s and the writer's results.

Among *Koch*'s strain there must have been representatives both of *M. tuberculosis* and *M. bovis* (cf. sixth experiment). It is impossible to understand that strains isolated from man could provoke fatal infection in voles, including development of greatly enlarged, caseous lymph glands and tubercles in liver, spleen and lungs. Not even 8.5×10^6 viable units of a highly virulent strain of *M. bovis* resulted in similar findings in the present study.

A very large infection dose might be assumed to mask the difference in the effect of the two species. However, the doses used by *Koch* cannot have been particularly large, as appears clearly from the survival times of the animals.

Finally *Koch's* voles may not have been *Microtus arvalis* but another member of the vole family. However, neither the closely related *Microtus agrestis* (field vole) (*Wells* 1938, *Griffith* 1937, 1939, 1941) nor other voles examined hitherto have shown pronounced susceptibility to *M. tuberculosis*.

The conclusion to be drawn from the present study is that *Microtus arvalis* is susceptible to *M. bovis* but extremely resistant to *M. tuberculosis* and thus, together with other voles, can be used for differentiation between the two species.

REFERENCES

Bize J. D., Étude du diagnostic différentiel entre les types humain et bovin du bacille tuberculeux. Utilisation dans la méthode biologique du campagnol roussâtre comme nouvel animal test. Thèse, Porrentruy 1961

Griffith A. S. Experimental tuberculosis in field voles and mice. Vet. Rec. *49* 982–984 1937

Griffith A. S., The relative susceptibility of the field-vole to the bovine, human and avian types of tubercle bacilli and to the vole strain of acid-fast bacillus (*Wells* 1937). J. Hyg. (Lond.) 39 244–259 1939.

Griffith A. S. Further experiments on the field vole with tubercle bacilli. J. Hyg. (Lond.) *41* 250–259 1941

Hauduroy P. & Bize J., Sensibilité et résistance du "campagnol roussâtre (*Clethrionomys glareolus Schreb.*) aux types bovin et humain du bacille tuberculeux. Ann. Inst. Pasteur 92 836–838, 1957

Jespersen A., Immunity to tuberculosis in red mice (*Clethrionomys g. glareolus Schreb.*) Acta path. microbiol. scand. *34* 87–96, 1954.

Jespersen, A., Infection of *Arvicola terrestris* (water rat) with *M. tuberculosis* and *M. bovis*. Acta path. microbiol. scand. Sect. B, *82* 667–675, 1974

Koch R., Die Ätiologie der Tuberkulose. Gesammelte Werke. Erster Band: 516, 537 541 Leipzig 1912.

Wells A. Q. The susceptibility of voles to human and bovine strains of tubercle bacilli. Brit. J. exp. Path. *19* 324–328, 1938.

path. microbiol. scand. Sect. B, 83 211–218, 1975

BACTERAEMIA IN RED MICE (*CLETHRIONOMYS G GLAREOLUS* SCHREB.) AFTER INTRAPERITONEAL INJECTION OF LARGE DOSES OF TUBERCLE BACILLI

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Jespersen, A. Bacteraemia in red mice (*Clethrionomys g glareolus* Schreb.) after intraperitoneal injection of large doses of tubercle bacilli. Acta path. microbiol. scand. Sect. B, 83 211–218, 1975.

Römer (1903) has demonstrated that white mice injected intraperitoneally with large doses of tubercle bacilli isolated from man survived longer than mice injected with tubercle bacilli isolated from cattle. The blood of the spontaneously dead animals contained large numbers of tubercle bacilli. In the present study red mice are injected intraperitoneally with 10 mg doses of different species of mycobacteria, and the number of bacilli in the blood is estimated at various intervals within the first 24 hours after the inoculation. The number of bacteria is considerably higher in the blood of mice injected with *M bovis* than in animals given *M tuberculosis* or BCG *M avium* is found in as large numbers as *M bovis* but, in contrast to *M bovis* *M avium* disappears rapidly from the blood stream. Supplementary experiments show that red mice injected with *M bovis* have a shorter survival time than mice injected with *M tuberculosis* and that the bacteraemia induced by *M bovis* into white mice is clearly less pronounced than in red mice.

Key words Tubercle bacilli; bacteraemia red mice; intraperitoneal injection.

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In experiments on the evaluation of the virulence of various strains of tubercle bacilli in animals, *Römer* (1903) found that white mice injected intraperitoneally with 10 mg culture of strains from tuberculous human subjects generally died after two to four weeks. In contrast, mice injected with strains from tuberculous cattle succumbed with symptoms of septicaemia a few days after the injection. Autopsy of the animals in both groups revealed tubercle bacilli in the blood either located freely or phagocytized by leucocytes. Since red mice are resistant to *M tuberculosis* but susceptible to *M bovis* (*Jespersen* 1954) in contrast to white mice which are resistant to both species, the difference observed by *Römer* might possibly be accentuated if red mice were used instead of white mice. The aim of the present paper was to study the bacteraemia during the first 24 hours after injection of large doses of various species of mycobacteria into red mice. Furthermore, the survival times in groups of mice similarly infected were also examined.

MATERIALS AND METHODS

Experimental 10 mg culture of *M tuberculosis* *M bovis* *M avium* or BCG were injected intraperitoneally into groups of 15 red mice. One animal from each group was killed after various intervals of from 0 to 8 hours four animals were killed after 24 hours. Smears were made from blood and the tubercle bacilli were counted after staining by the Ziehl-Neelsen method.

Supplementary experiments. (a) Examination of the survival times of red mice infected with strains of *M tuberculosis* and *M bovis* Groups of 10 animals were injected intraperitoneally with 10 mg culture of a virulent strain of *M tuberculosis*, a virulent strain of *M bovis* an attenuated strain of *M bovis* and a BCG vaccine. The animals were observed until they died spontaneously (b) Comparison of the bacteraemia in red mice and white mice Two groups of six white mice and two groups of three red mice were injected intraperitoneally with 10 mg of a strain of *M tuberculosis* and *M bovis* respectively The animals were killed after 2, 4 and 24 hours, and the bacteria were counted in smears from blood.

Strains All the strains—also the strain of *M avium*—were isolated from man. Virulence determination prior to the experiment was carried out on the usual experimental animals and showed the virulence characteristic of the species concerned.

Bacterial suspensions for infection *M tuberculosis* and *M bovis* were cultured in Bezredka fluid medium. The sediment was collected by a pasteur pipette, dried by pressing with filter paper weighed, and ground in mortar Saline was added until the suspension contained 10 mg culture per ml. The suspension of *M avium* was made in a similar way after harvesting the colonies from a 6-week-old Löwenstein-Jensen culture.

Blood sampling and preparation of smears. The mice were anaesthetized with ether, placed in supine position on a wooden tray and fixed with pins through the front and hind legs. From a small transverse cut into the skin of the lower part of the sternum, the skin over the thorax and right front leg was loosened and clipped The skin in the axilla was then loosened, thus exposing the axillary vessels and forming a pouch for the collection of the blood. The vessels were cut and, when the blood had gushed out for while, 25 µl was collected in a pipette. The blood was placed on a polished slide and smeared out by passing the pipette across the slide using regular movements. In this way the layer was thicker than it would have been if a slide had been used. During sampling at intervals p to 4 minutes, the mouse

TABLE 1 *N f Tubercle Bacilli in Smear of Blood from Axillary Vessels Taken at Various Times after Intraperitoneal Injection of 10 Mg Culture of* M. bovis *per Red Mouse*

No. of bact. per sight field	0 m	1 m	2 m	4 m	8 m	15 m	½ h	1 h	2 h	4 h	8 h	24 h	24 h	24 h
							Non-phagocytized bacteria							
0	100	98	99	96	96	99	57	56	2	4	0	0	0	0
1		2		3		1	28	11	10	2				1
2–3			1	1	2		10	19	30	4			1	
4–9							5	11	38	11	1	1	5	8
10–25								3	20	38	87	75	53	57
26–100										21	16	24	39	34
							Phagocytized bacteria							
0	100	100	100	100	100	100	100	100	97	98	100	97	94	[illegible]
1									1	1		1	3	6
2–3												2	3	5
4–9									2					2
10–25										1				1
26–100														

The figures stated below the various times give the number of sight fields containing 1 2 3 bacteria, etc.
m = minutes h = hours.

s anaesthetised, the skin was removed from the domen, thorax and axilla, and the bacterial suspension was injected. '0 minutes' indicates that the illary vessels were cut immediately after injection the suspension.

As soon as the slides had lost their moist appearance, they were placed in 5 per cent formaldehyde in physiological saline for 24 hours. The ythrocytes were rapidly haemolysed, while the icocytes retained their normal form. The tubercle cilli were stained by the Ziehl-Neelsen method.

Counting of tubercle bacilli. Using an oil immersion lens 100 × the tubercle bacilli were unted in sight fields distributed over three ngths of the slide. The non-phagocytised tubercle cilli were counted in the first 100 sight fields, bsequently the phagocytised bacteria were count l in a further 100 sight fields.

RESULTS

acteraemia

M bovis (Table 1) The figures stated below the various times give the number of ght fields containing 0 1 2–3 bacteria, etc.

Non-phagocytised tubercle bacilli could be emonstrated as early as 1 minute after the jection. The number increased rapidly fter ? hours, bacteria could be seen in almost all sight fields in one-fifth of the fields ≥10 bacteria were in evidence After 8 and 4 hours, all sight fields contained bacteria n more than 90 per cent 10–100 bacteria.

Phagocytised tubercle bacilli could not be lemonstrated until after 2 hours. The numer was small and the highest number was een after 24 hours. All the bacteria were ound in polynuclear leucocytes.

The bacteria in the blood smears were ither located singly or in units of varying izes. In order to gain an idea of the size of he units, the number of bacteria per unit was counted in the blood taken after 4 and 24 hours (*Table 2*) By way of comparison, differential counting of the units in the inoculated Besredka culture was made. The Besredka culture contained 9 units with ≥10 bacteria, including one or two very large units. In blood taken after 4 hours, only one unit with ≥10 bacteria was found and, after 24 hours, no units of that size were in evidence In the latter smear the bacteria were

TABLE 2. *N of Bacteria per Unit in Besredka Culture and in Smears of Blood Taken 4 and 24 H after Intraperitoneal Injection of 10 Mg f the Culture in Question*

Bacteria per unit	Besredka culture	Axillary blood 4 h	24 h
1	63	70	32
2	16	15	44
3–9	12	14	24
10–25	6	1	
26–100	2		
>100	1		

long and single bacteria were relatively few relatively many units contained 2 and 3 bacteria, thus showing that the bacteria had already started to multiply

BCG (Table 3) Non phagocytised bacteria could be seen for the first time after 4 minutes. The number might vary but generally positive sight fields were few only and the units contained few bacteria. There was no tendency to an increase during the interval between the first hours after injection until the next day

The number of phagocytised bacteria was relatively large for instance, there were almost equally many phagocytised and non-phagocytised bacteria after 1 and 24 hours. A single unit phagocytised by a mononuclear cell was seen after 24 hours, while all the others were found in polynuclear cells.

M tuberculosis (Table 4) The first non phagocytised tubercle bacilli were found after 8 minutes when 1–3 bacteria were seen in 17 sight fields. Between 30 minutes and 8 hours, a few bacteria could still be seen in a few sight fields, but there was no definite tendency towards an increase or a decrease. After 24 hours, the number of bacteria in three of the mice was smaller than that on the previous day in the fourth mouse, how ever bacteria were observed in about one third of the sight fields. Phagocytised bacteria, all in polynuclear leucocytes, were seen for the first time after 15 minutes the number was smaller than that of the non-phagocytised bacteria, but relatively large as ...

TABLE 3 *Bacteraemia in Red Mice 0–24 H after Intraperitoneal I jection of 10 Mg BCG*

	0 m	1 m	2 m	4 m	8 m	15 m	½ h	1 h	2 h	4 h	8 h	24 h	24 h	24 h	[illegible]
								Non phagocytized bacteria							
0	100	100	100	91	95	54	64	79	100	75	80	100	100	95	[illegible]
1				7	3	17	22	15		15	5			1	
2–3				1	2	25	10	5		5	6			3	
4–9				1		6	4	2		1	8			1	
10–25								1		3	1				
26–100										1					
								Phagocytized bacteria							
0	100	100	100	100	100	99	100	82	100	93	90	100	100	98	[illegible]
1								9			3			1	
2–3								5		2	4			1	3
4–9						1		3			1				3
10–25								1		5	2				1
26–100															

m = minutes h = hours.

TABLE 4 *Bacteraemia in Red Mice 0–24 H after Intraperitoneal Injection of 10 Mg* M. tuberculosis

	0 m	1 m	2 m	4 m	8 m	15 m	½ h	1 h	2 h	4 h	8 h	24 h	24 h	24 h	24 h
								Non-phagocytized bacteria							
0	100	100	100	100	83	97	86	92	86	96	99	99	99	65	[illegible]
1					11	3	9	7	11	3	1	1		16	
2–3					6		4	1	2	1				13	
4–9							1		1				1	6	
10–25															
26–100															
								Phagocytized bacteria							
0	100	100	100	100	100	99	99	93	96	94	97	98	98	95	97
1						1	1	5	4	4	2	1		2	1
2–3								1			1	1	2	1	
4–9								1		1					
10–25										1				2	
26–100															

m = minutes h = hours.

pared with the number of phagocytized *M bovis* (Table 1)

M avium (Table 5) The first non-phagocytized bacteria were found after 2 minutes. After 15 minutes, bacteria were observed in all sight fields in about half the fields there were 26–100 bacteria. The bacteraemia culminated after 2 hours when one-fifth of the sight fields contained >100 bacteria upon which a sharp decrease in the number of bacteria occurred After 24 hours, a few bacteria in three of the mice could be found in a few sight fields. As regards the fourth mouse bacteria were observed in about every third

TABLE 5. *Bacteraemia in Red Mic 0–24 H after Intraperitoneal Injection of 10 Mg* M. avium

	0 m	1 m	2 m	4 m	8 m	15 m	½ h	1 h	2 h	4 h	6 h	24 h	24 h	24 h	24 h
								Non phagocytized bacteria							
0	100	100	91	84	61	0	0	0	0	9	97	100	93	71	99
1			5	11	15		1			9	2		3	5	1
2–3			4	5	11		1			22	1		1	14	
4–9					11	3	3			39			3	9	
-25					1	51	67	36	3	21				1	
100					1	46	28	64	78						
100									19						
								Phagocytized bacteri							
0	100	100	100	100	100	100	93	100	76	86	71	96	95	85	97
1							4		3	5	6	3	3	5	2
2 3							3		1	2	5		1	1	1
4–9									1		4	1		1	
-25									6	6	10		1	5	
-100									13	1	4			3	
-100															

m = minutes h = hours.

ight field, only one of which contained ≥10 bacteria.

Phagocytized bacteria were not seen until after 30 minutes. The number was considerably larger than that of phagocytized *M bovis.*

Comparison of the Bacteraemia in White and Red Mice

Table 6 shows the results of bacterial counts in smears of blood taken 2, 4 and 24 hours after intraperitoneal injection of 10 mg *M tuberculosis* or *M bovis* into white or red mice.

In the white mice injected with *M tuberculosis* or *M bovis* and in the red mice injected with *M tuberculosis* there were either no or only few bacteria in a few sight fields. The findings in red mice injected with *M bovis* were quite different and the same as those reported previously as early as 2 hours after the injection there were tubercle bacilli in about half of the sight fields and after 4 and 24 hours in all sight fields 90 per cent of which contained ≥10 bacteria.

Survival Times of Red Mice Injected with Large Doses of M. tuberculosis *or* M. bovis

Table 7 shows the survival times in days of groups of 10 mice injected intraperitoneally with 10 mg of different mycobacterial strains. In the group injected with the virulent strain of *M bovis* (T 5605 B) the median survival times were 2 days, being 15.5 days in the group injected with an attenuated strain of *M bovis* (V 19647) and >90 days in the group injected with BCG. As regards mice injected with two virulent strains of *M tuberculosis* (E 10883 H and E 6879 H) the median survival time was 12 days in both groups.

DISCUSSION

Bacteraemia. Tubercle bacilli injected into the peritoneal cavity pass through the lymphatic vessels in the diaphragm, and via the ductus thoracicus to the venous system. In the lungs, a great number of the bacteria are phagocytized, but some of the large bacterial units are retained mechanically for a time in the capillaries. Differential counting of a

TABLE 6. *Bacteraemia in White and Red Mice 2–24 H after Intraperitoneal I jection of 10 Mg* M. bovis *or* M. tuberculosis

	white mice						red mice		
	2 h		4 h		24 h		2 h	4 h	24 h
	M bovis 10 mg i.p.								
0	87	97	96	91	100	100	56	0	0
1	7	3	3	6			10		
2–3	6		1	3			18	1	
4–9							9	8	6
10–25							4	73	59
26–100							1	18	35
>100							2		
	M tuberculosis 10 mg i.p.								
0	100	99	100	100	100	100	93	98	97
1		1					5	1	
2–3							2	1	2
4–9									1
10–25									
26–100									
>100									

m = minutes h = hours.

TABLE 7 *Survival Times in Days of G oups of Red Mice Injected I t aperitoneally with 10 Mg of Different Strains f Mycobacteria*

Virulent *M bovis*	Virulent *M tuberculosis*		Atten. *M bovis*	BCG
T 5605 B	E 10883 H	E 6879 H	V 19647	
1	4	3	4	7
1	7	4	6	45
1	7	5	7	>90
1	8	7	10	>90
2	9	12	13	>90
2	15	12	18	>90
2	15	16	21	>90
3	27	20	35	>90
3	27	24	>90	>90
26	66	29	>90	>90

Underlined figures = median survival time.

suspension of *M bovis* used for injection and of a smear of blood made 4 hours after injection showed a smaller number of large units in the smear than in the bacterial suspension. A transient mechanical retention of bacterial units in the lung capillaries has been demonstrated in an experiment where hamsters were injected intraperitoneally with BCG grown in Dubos fluid medium (*Jespersen & Bentzon* 1964) Tubercle bacilli that pass through or are released secondarily from the lung capillaries become phagocytized by RES cells, primarily in the liver and spleen.

The tubercle bacilli in the peritoneal cavity

pass through the lymphatic vessels to the blood, as non-phagocytized as well as phagocytized bacteria. All experiments, independent of the bacterial species used, show in blood smears first the non-phagocytized bacteria and later the bacteria phagocytized by polynuclear cells. The relative number of phagocytized bacteria is greater in animals injected with *M avium*, *M tuberculosis* and BCG than in those given *M bovis*.

The mechanism of the bacteraemia induced by the individual species of mycobacteria is difficult to explain because of the enormous number of bacteria injected. The sparse accumulation of *M tuberculosis* in the blood may be due to the fact that these bacteria are retained in the peritoneum to a greater extent than *M bovis* and *M avium*. Another explanation may be that the two latter species, but not *M tuberculosis* contain a factor that prevents them from being caught by the RES cells. Preliminary experiments support the first theory. The massive bacteraemia after 24 hours seen only after injection of *M bovis* is undoubtedly due to the fact that the cells of the RES system have gradually become saturated with bacteria which then accumulate in the blood.

There seems to be a correlation between bacteraemia and the virulence of the bacteria for the animals in question. This is evident as regards *M bovis*, BCG and *M tuberculosis* but applies also to *M avium* when the evaluation is based on the bacteraemia after 24 hours. In spite of the pronounced bacteraemia seen during the first hours after injection, red mice injected with large doses of *M avium* survive just as long as those given *M tuberculosis* (*Jespersen* unpublished). There also seems to be a relationship between virulence and the number of phagocytized bacteria so that the virulence is higher the fewer phagocytized bacteria.

The massive bacteraemia induced by *M bovis* in red mice cannot be induced in white mice by injection of either *M tuberculosis* or *M bovis*. As mentioned the red mouse is strongly susceptible to *M bovis* infection (*Jespersen* 1954) while the white mouse is resistant to both *M tuberculosis* and *M bovis*.

Survival times. Römer's first assumption was that the survival times of mice injected with large doses could be used for differentiation of tubercle bacilli isolated from man and cattle. However he abandoned the idea when he found that one of the strains isolated from tuberculous patients provoked an infection which was just as acute as that caused by strains isolated from tuberculous cattle.

In the present study the median survival times of red mice injected with a virulent strain of *M bovis* and two virulent strains of *M tuberculosis* were 2, 12 and 12 days, respectively. Subsequent experiments using a total of about 30 strains showed that the median survival times were consistently shorter in groups of animals injected with *M bovis* than in those given *M tuberculosis* but that the difference was not always as pronounced as in the present study. Animals injected with *M bovis* survived for a few days only and some died during the first 24 hours after the injection. The survival times of animals injected with *M tuberculosis* were longer and more varied, and deaths never occurred during the first 24 hours.

The median survival time cannot be used to distinguish between virulent strains of *M tuberculosis* and attenuated strains of *M bovis*. However there seems to be a difference in the distribution of the survival times which can be used in differential diagnosis. All the animals in a group injected with a virulent strain of *M tuberculosis* will die within a relatively short period but among animals injected with an attenuated strain of *M bovis* there will be a few which will survive the infection.

On the other hand, within a given species of mycobacteria the median survival time using the 10 mg test, can be used as indication of the virulence of a strain. The median survival times of groups of mice injected with a virulent strain of *M bovis* an attenuated strain of *M bovis* and BCG were 2, 16 and >90 days, respectively.

REFERENCES

Jespersen A Immunity to tuberculosis in red mice (Clethrionomys g. glareolus Schreb.) Acta path. microbiol. scand. *34* 87–96 1954

Jespersen, A. & Bentzon M W The virulence of various strains of BCG determined on the golden hamster Acta tuberc. scand. *44* 222–249 1964

Römer P H Über Tuberkelbacillenstämme verschiedener Herkunft. Beitr Exp. Ther. *6* 1–110 1903

Acta path. microbiol. scand. Sect. B, 83 219–225 1975

STAPHYLOCOCCUS AUREUS IN CYSTIC FIBROSIS ANTIBIOTIC SENSITIVITY AND PHAGE TYPES DURING THE LATEST DECADE

Investigations of the Occurrence of Protein A and some other Properties of Recently Isolated Strains in Relation to the Occurrence of Precipitating Antibodies

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Hoff G. E. & Høiby N. *Staphylococcus aureus* in cystic fibrosis antibiotic sensitivity and phage types during the latest decade. Investigations of the occurrence of protein A and some other properties of recently isolated strains in relation to the occurrence of precipitating antibodies. Acta path. microbiol. scand. Sect. B, 83 219–225 1975.

During the recent decade, 1651 isolates of *Staphylococcus aureus* from 111 patients with cystic fibrosis have been tested for antibiotic sensitivity and half of the isolates have been phage typed. All the patients were followed in one clinic and the policy of antibiotic treatment was consistent during this period. The results show a dynamic situation where 'epidemic' phage types during recent years has been gradually replaced by other types and, during the same period, the prevalence of strains resistant to more than one antibiotic decreased. Multiresistant strains including strains resistant to methicillin were infrequent in these patients From 23 per cent of the patients, the same strains were repeatedly isolated for more than 1 year despite an apparently successful chemotherapy Recently isolated strains were found to produce cell-bound as well as extracellular protein A. Ninety-one per cent of the strains produced extracellular lipase and only 8 per cent were resistant to mercury chloride. Eighty-one per cent of the patients produced precipitating antibodies against *S aureus* as judged by crossed immunoelectrophoresis. The investigated properties of *S aureus* were not significantly correlated with the occurrence of precipitating antibodies against these bacteria. The possible significance of protein A in the pathology of the respiratory tract infection is discussed.

Key words *S aureus* cystic fibrosis antibiotic sensitivity; phage types.

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The importance of *Staphylococcus aureus* (*S aureus*) in cystic fibrosis (CF) has been recognized since the first reports concerning this disease appeared (1) but despite chemotherapy and prolonged survival of the patients (pts.) *S aureus* remains a main pathogen in CF (7 12 13 15, 17 19 20, 25–27)

The reason why *S. aureus* is closely associated with CF remains obscure although several explanations have been offered (7, 12, 13, 15, 19, 25).

It applies to most of the published reports on *S. aureus* in CF that observations have been limited to cover short periods of time and little is known about fluctuations in the properties of *S. aureus* isolated from CF pts. during longer periods. According to a countrywide study fluctuations as regards phage type and antibiotic resistance pattern of *S. aureus* occur in Danish hospitals (4, 22, 28).

Intensive CF treatment in Denmark has largely been centralized to one clinic during the recent decade. The bacteriological service has been carried out by one laboratory in close co-operation with the clinicians, and the principles and policy of chemotherapy have been consistent during this period.

The aim of the present study is to report the changes in antibiotic resistance and phage type pattern of *S. aureus* isolated from CF pts. who have been followed in the CF Clinic of Rigshospitalet, Tagensvej. In addition, strains of *S. aureus* recently isolated from CF pts. have been investigated for some properties possibly associated with pathogenicity: protein A production, lipase production, and resistance to mercury chloride (4, 6, 8–11, 22, 30) and sera from patients have been examined for the occurrence of precipitating antibodies against *S. aureus*.

MATERIALS AND METHODS

Patients

During the period January 1st 1965 to June 30th 1974 *S. aureus* has been isolated in 1651 specimens obtained from the respiratory tract of 111 CF pts. The pts. were followed as outpatients and were examined every or every other month in the CF Clinic, if necessary they were hospitalized (14, 15). The number of pts. with staphylococci and the number of isolates per year were: 2/6, 6/19, 11/35, 11/44, 23/77, 50/240, 64/345, 75/400, 82/345 and during the first 6 months of 1974: 71/191. The results obtained during the first 5 years will be lumped together.

Bacteriological Examinations

At each visit to the clinic and regularly during admissions, sputum or tracheal secretion was examined bacteriologically including primary as well as secondary testing of antibiotic sensitivity as described previously (15). Doubtfully methicillin-resistant strains were examined at 30° C and on media including 7.5 per cent NaCl (3). The present study concerns the sensitivity to the following antibiotics: penicillin (low dose), streptomycin, tetracyclin, methicillin, fusidic acid, erythromycin and rifampicin. Fourty-seven per cent of the isolates were phage typed in the Department of Hospital Infections, Statens Seruminstitut, using the method of *Blair & Williams* (1961) with the phages 84, 85, 89; the experimental phage 6557 being added to the set (29). Definitions of terms used: *80*: types within the 52, 52A, 80, 81-complex. *GI-other*: other types and complexes within phage group I. *GII*: types and complexes within phage group II. *83A*: types within the 83A, 84, 85, 6557, 89-complex. *GIII-other*: other types and complexes within phage group III. *GIV*: pure type 42D. *MISC*: 187 and 94. *MIX G.*: strains lysed by phages from more than one group. *NT*: Non-typable strains.

During the last 4 months of 1973, sera and strains of *S. aureus* were simultaneously obtained from 53 of the CF pts. The strains were examined for antibiotic sensitivity, phage type and protein A production. Information about production of extracellular lipase and resistance to mercury chloride was supplied by dr. *K. Rosendal*, Statens Seruminstitut.

Examination of Protein A Production in S.

Protein A production was investigated according to *Winblad & Ericson* (1973) with modifications of the method to a human system. Fresh human O Rh CCDee red blood cells (RBC) in ACD solution and human anti-D (IgG_1) serum were supplied by dr. *D. Jerne*, Statens Seruminstitut. The RBC were washed in phosphate buffered saline (PBS) pH 7.38, mixed with anti-D serum and PBS (1:1.3) and incubated for 1.5 h at 37° C, washed and resuspended to 5 per cent (v/v) in PBS. Coombs direct test including control with non-sensibilized RBC was done as a control of the sensibilization. Protein A positive control strain: Cowan I *S. aureus* 4972 and purified protein A (21). Negative controls: Wood 46 and a coagulase negative *S. epidermidis*. The *S. aureus* control strains were supplied by dr. *J. Lind*, and lyophilized protein A by dr. *K. Jensen*, Statens Seruminstitut.

Slide agglutination test for cell-bound protein A: the strains were cultivated overnight at 35° C on a peptone-rich solid medium (Trieber (14)). A small amount of the culture was mixed with one

op of 5 per cent sensitized RBC on a glass slide d examined for 5 min during tilting of the glass. sitive results were observed as haemagglutina- n.

Tray agglutination test f r extracellular protein in broth supernatant Overnight broth cultures re centrifuged t 48,200 × g for 20 min. One op of the supernatant was mixed with one drop 5 per cent sensitized RBC in the wells of a pla- c haemagglutination tray incubated for 2 h at ° C, and read.

Tray aggluti ation test for quantitation of ex ected protein A. 0.50 g of overnight *S aureus* ltures (Tröche) was suspended in 10.0 ml phos- ate buffer 1/15 M pH 5.9 and extracted quan- tatively for protein A at 100° C for ½ h accord- g to *Jensen* (1959) The cells were removed by ntrifugation at 48,200 × g for 20 min and one rop of 5 per cent sensitized RBC was mixed with e drop of the supernatant in two-fold dilutions th PBS and incubated and read as described ove. All the quantitations were done simultane- sly

recipitating A tibodies against S. aureus

The number of precipitating antibodies against *aureus* was studied by means of crossed immuno- lectrophoresis in a microtechnique as described reviously (16) using a mixture of ultrasonically repared antigens (18) of 4 *S aureus* strains re resenting the 4 phage groups (protein concen- ration 8.6 g per litre) Thirty-three per cent of era from normal children contain one weak preci- itin against this antigen sample (unpublished re- ults) Protein A precipitates were not counted.

Chemotherape tic Treatment (Selective Pressure S. aureus)

A combination of oxacillin + fusidic acid given orally for 14 days is routinely used for anti-sta- phylococcal treatment, unless the strain was resi- stant to one of these drugs If so this drug is re placed by rifampicin. If the strain is sensitive to penicillin, V-penicillin replaces oxacillin in the combination in some instances, methicillin inhala- tion is added and the oxacillin treatment extended for some months prophylactically On an average, each pt. receive 2 courses of anti-staphylococcal treatment per year and in addition 0.5 courses of treatment with aminoglycosides or penicillin deri- vates against other bacteria (15) Treatment was only given if indicated by clinical and bacteriologi- cal examinations (15)

Statistical Methods (5)

The χ^2-test with Yates correction if indicated.

RESULTS

The results of antibiotic sensitivity testing are given in Tables 1 and 2. It is seen that the majority of strains were resistant to peni- cillin only and that the majority of pts. had harboured such strains or fully sensitive strains, whereas multiresistant strains were rare. However 12.6 per cent of the pts. have at some time harboured methicillin resistant strains, whereas only 5 per cent and 6 per

TABLE 1 *Distribution on Antibiograms of* Staphylococcus aureus *from Patients with Cystic Fibrosis 1965–1974*

	Percentage of strains						
	1965–69 (181 strains)	1970 (240 strains)	1971 (343 strains)	1972 (400 strains)	1973 (345 strains)	1974 (191 strains)	Total (1651 strains)
Sensitive	8	14	8	14	18	18	15
P	57	52	62	64	60	76	60
PS	21	8	11	5	6	1	15
PT	7	10	4	2	3	1	10
PST(E)(R)	4	8	4	7	8	2	7
PR	5	3	3	2	1	0	2
PSTM(E)(R)(F)	2	1	4	4	1	1	2

Code Strains resistant to penicillin (P) streptomycin (S) tetracyclines (T) rifampicin (R) methi- cillin (M) erythromycin (E) fusidic acid (F) PSTM(E)(R)(F) indicate strains resistant to PSTM and possibly even to one or more of the antibiotics in brackets. Only the most common antibiograms are included covering more than 95 per cent of the isolates. As patients can be infected with more than one strain per year the sum amounts t more than 100 per cent.

TABLE 2. *Percentage of Patients with Cystic Fibrosis Harbouring* Staphylococcus aureus *Classified According to Antibiograms 1965–1974*

	Percentage of patients harbouring the strains						
	1965–69 (27 patients)	1970 (50 patients)	1971 (64 patients)	1972 (75 patients)	1973 (82 patients)	1974 (71 patients)	Total (111 patients)
Sensitive	30	26	25	33	29	28	47
P	70	66	80	88	77	85	92
PS	15	8	13	8	13	3	8
PT	11	16	8	3	5	1	4
PST (E) (R)	22	10	6	11	10	4	18
PR	4	6	3	3	1	0	4
PSTM (E) (R) (F)	11	4	5	5	4	1	10

cent have harboured strains resistant to fusidic acid or rifampicin (these values include strains with antibiograms not given in the Tables). Moreover the sensitivity patterns of *S. aureus* have changed toward increased sensitivity during the latest years ($p < 0.0005$).

The distribution of *S. aureus* on the different phage groups and complexes and the relationship between these and the antibiograms (Table 3) shows that no single group or complex predominated, and that the epidemic complexes' (*80'* and *83A*) were found in significant numbers in CF pts. However these epidemic complexes'—including most of the strains resistant to agents other than penicillin—have steadily decreased in prevalence during the latest years and have been replaced by other groups and complexes ($p < 0.01$). Strains belonging to phage group IV were not isolated from the pts. Only 47 per cent of the strains have been phage typed, but these strains were not found to differ from the remaining strains as regards prevalence of the various antibiograms.

It applies to 25 of the pts. (23 per cent) that the same strains (same phage type and antibiogram) were repeatedly isolated throughout more than one year (range 1 to 8 years) although the infections were apparently interrupted as the strains intermittently disappeared during and after chemotherapy. This series of 25 patients included four pairs of CF siblings—each pair carrying the same strain—as compared with 4 pairs in the remaining series of 86 pts. ($p < 0.005$). Twelve of these repeatedly isolated strains were resistant to agents other than penicillin and 11 of these belonged to the epidemic complexes.

TABLE 3. *Phage Types and Relationship between Phage Types and Antibiograms of Staphylococcus* aureus *from Patients with Cystic Fibrosis 1965–1974*

	Percentage of strains (767 strains)	Percentage of patients harbouring the strains (111 patients)				
		Sensitive	P	PS	PST (E) (M) (R) (F)	Other antibiograms
80'	15	11	14	9	7	3
G.I.-other	8	9	14	3	0	1
G II	24	10	31	2	1	3
'83A	11	1	15	0	15	4
G.III-other	16	4	3?	2	3	8
MISC. + MIX. G.	18	4	32	4	2	13
NT	8	8	16	1	3	3

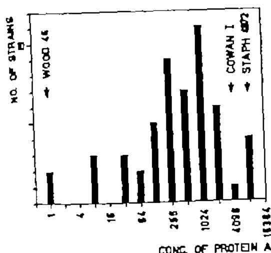


Fig 1. Quantitation of extracted protein A from 53 strains of *S aureus* isolated from 53 patients with cystic fibrosis. Concentration of protein A is given as the reciprocal of the highest dilution resulting in haemagglutination of sensitized human red blood cells. The values obtained with negative (WOOD 46) and positive (COWAN I & STAPH. 4972) controls are indicated by arrows.

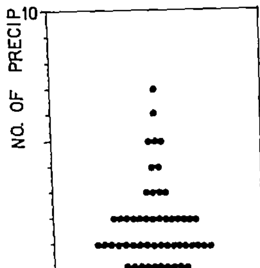


Fig 2. Number of precipitating antibodies against *S aureus* (NO. OF PRECIP.) in sera from 53 patients with cystic fibrosis. Each patient is indicated by a dot.

80 and *83A*, this accumulation of resistant epidemic strains is significant ($p<0.05$).

The investigations into the occurrence of protein A in 53 strains of *S aureus* showed that all the strains produced cell-bound as well as extracellular protein A. The results of quantitation of extracted protein A are given in Fig. 1. Nine per cent of the strains contained as much protein A as Cowan I or more and 4 per cent contained as little as Wood 46. None of these 53 strains were methicillin resistant.

Only 4 (8 per cent) of the strains were resistant to mercury chloride; these 4 strains also produced extracellular lipase and were resistant to penicillin and streptomycin; three of the latter belonged to the *80* complex. Fourty-eight (91 per cent) of the strains produced extracellular lipase; they were found in all of the phage groups.

Fourty-three (81 per cent) of the 53 CF pts. had pre itating antibodies against *S aureus* and 5 of these had 2 or more precipitins against his bacteria (Fig. 2). There were no signif nt correlations between the precipitins and the studied properties of *S aureus* isolated from the pts. and apart from the correlations mentioned above these properties were not otherwise mutually correlated.

DISCUSSION

The results show that *S aureus* from CF pts. belong to a wide range of types as regards the properties investigated. In accordance with earlier reports, these pts. seem susceptible to epidemic as well as non-epidemic strains (7, 13, 19, 27). Frequently the same strain is isolated repeatedly from a pt. during prolonged periods of time in spite of apparently successful chemotherapy. These repeatedly isolated strains often belong to epidemic types resistant to several antibiotics, and CF siblings are often simultaneously infected with such strains.

Even though the use of the combination of cloxacillin and fusidic acid has predominated throughout 10 years, strains resistant to these 2 drugs—including multiresistant strains belonging to the epidemic phage complexes—have only represented a minor even decreasing problem in cases of CF pts.

In accordance with this, most of the 53 strains investigated were sensitive to mercury chloride and produced extracellular lipase (4 22) Although the results of our investigation are not quite comparable with those obtained in a nation wide study of bacteremia in Denmark, the changes in phage types and antibiotic sensitivity are correlated with similar changes towards more sensitive types of *S aureus* and a decrease in the prevalence of epidemic complexes during the same period studied on a country wide basis (28) The *S aureus* strains included in the present study are, however more similar to strains aquired outside hospitals than to hospital strains, considered on a country-wide basis, as regards phage types as well as antibiograms (28) This is not surprising considering that CF pts. are mainly treated as outpatients in our clinic.

In accordance with other authors (12 19) it is our experience that CF pts. do not contract infections with *S aureus* or other bacteria at sites other than the respiratory tract irrespective of the frequent pulmonary infections. The reason for this is unclear but might be related to the pronounced humoral immune response against *S aureus* and other bacteria (Fig. 6) (7 12, 16 17 20 25 26)

Protein A was demonstrated in all strains of *S aureus* examined in accordance with other reports on non-CF *S aureus* (8, 21 23 24 31) In the present study human IgG_1 was used instead of rabbit immunoglobulin to demonstrate protein A. It is therefore obvious that all the *S aureus* strains investigated for protein A production could react with the Fc part of human IgG Such reactions have been shown to promote inflammation (10 11 30) and to interfere with phagocytosis (6 9) The reactions between protein A and IgG could therefore possibly be the main reason why *S aureus* is one of the predominating pathogens in CF (15)

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REFERENCES

1 *Andersen D H.*, Cystic fibrosis of the pancreas and its relation to celiac disease Am. J Dis. Child. 56 344–399 1938.

2. *Blair J E. & Williams R. E. O.*, Phage typing of staphylococci. Bull. Wld. Hlth. Org 24: 771 784 1961

3 *Brown D F J & Kothari D*: The reliability of methicillin sensitivity tests on four culture media. J clin. Path. 27 420–426, 1974

4 *Bülow P.*, Staphylococci in Danish hospitals during the last decade Factors influencing some properties of predominant epidemic strains. Ann. N Y Acad. Sci. *182* 21 39 1971

5 *Diem K.* (Ed.): Documenta Geigy Scientific Tables. J R. Geigy S. A., Basel 1962, p 36-39.

6. *Dossett J H Kronvall G Williams, R. C Jr & Quie P G* Antiphagocytic effects of staphylococcal protein A. J Immunol. *103* 1405–1410, 1969

7 *Eichenwald H F.*, Staphylococcal infection in cystic fibrosis. In McIntosh, R. (Ed.) Research on Cystic Fibrosis. The French-Bray Printing Co., Baltimore, Maryland 1960, p. 89-120.

8. *Forsgren A.*, Significance of protein A production by staphylococci Infect. and Immun. *2* 672–673 1970

9 *Forsgren A. & Quie P G* Effects of staphylococcal protein A on heat labile opsonins. J Immunol. *112* 1177–1180 1974

10. *Gustafson G T Sjöquist J & Stålenheim, G* Protein A from *Staphylococcus aureus.* II Arthus-like reactions produced in rabbits by interaction of protein A and human γ-globulin J Immunol. 98 1178-1181 1967

11 *Gustafson, G T Stålenheim G., Forsgren A & Sjöquist J* "Protein A from *Staphylococcus aureus.* IV Production of anaphylaxis-like cutaneous and systemic reactions in non-immunized guinea-pigs. J Immunol. *100* 530-534, 1968.

12. *Halbert S P di Sant Agnese P A & Reisman Kotek F* Staphylococcal antibodies in cystic fibrosis of the pancreas. Pediatrics *26* 792 799, 1960.

13 *Huang, N N Van Loon E. L. & Sheng, K T* The flora of the respiratory tract of patients with cystic fibrosis of the pancreas. J Pediat. *59* 51 521 1961

14 *Høiby N & Axelsen N H* Identification and quantitation of precipitins against *Pseudomonas aeruginosa* in patients with cystic fibrosis by means of crossed immunoelectrophoresis with intermediate gel. Acta path. microbiol scand. Sect. B, *81* 298–308, 1973

15 *Høiby* V Epidemiological investigations of the respiratory tract bacteriology in patients with

cystic fibrosis. Acta path. microbiol. scand. Sect. B, 82 541 550 1974
5. *Høiby N.: Pseudomonas aeruginosa* infection in cystic fibrosis Relationship between mucoid strains of *Pseudomonas aeruginosa* and the humoral immune response. Acta path. microbiol. scand. Sect. B, 82 551–558, 1974
7 *Høiby N & Wiik A* Antibacterial precipitins and autoantibodies in serum of patients with cystic fibrosis. In press. Scand. J Resp. Dis.
8. *Høiby N* Cross-reactions between *Pseudomonas aeruginosa* and thirty-six other bacterial species. In press. Scand. J Immunol.
9. *Iacocca V F Sibinga M S & Barbero G J:* Respiratory tract bacteriology in cystic fibrosis. Am. J Dis. Child. *106:* 315–324 1963
10. *Iacocca, V F & Barbero G J.,* Serum antistaphylococcal alphahaemolysin titres in cystic fibrosis. Arch. Dis. Childh. *43* 220–222, 1968.
11 *Jensen K.* Undersøgelser over stafylokokkernes antigenstruktur Thesis. University of Copenhagen, Munksgaard, Copenhagen 1959 p. 11–117
12. *Jessen O Rosendal, K., Bülow P F ber V & Eriksen, K. R.:* Changing staphylococci and staphylococci infections. A ten-year study of bacteria and cases of bacteriemia. N Engl. J Med. *281* 627–635 1969
13. *Kronvall G., Dossett J H., Quie P G & Williams R. C J* Occurrence of protein A in staphylococcal strains: quantitative aspects and correlation to antigenic and bacteriophage types. Infect. and Immun. *3* 10–15 1971
14. *Lind I* Correlation between the occurrence of protein A and some other properties in *Staphylococcus aureus*. Acta path. microbiol. scand. Sect. B, *80* 702–708, 1972.
25. *May J R., Herrick N C & Thompson, D.,* Bacterial infection in cystic fibrosis. Arch. Dis. Childh. *47* 908–913 1972.
26. *Mearns M B Hunt G H & Rushworth R.,* Bacterial flora of respiratory tract in patients with cystic fibrosis, 1950–1971 Arch. Dis. Childh. *47* 902–907 1972.
27 *Pittman, F E., Hsu C., Goode L. & di Sant' Agnese P A.,* Phage groups and antibiotic sensitivity of *Staphylococcus aureus* associated with cystic fibrosis of the pancreas. Pediatrics *24* 40–42, 1959
28. *Rosendal K., Bulow P Bentzon M V & Eriksen K R. Staphylococcus aureus* strains in Danish hospitals from Jan. 1 1966 to Dec. 31 1973 Manuscript in preparation.
29 *Rosendal, K. & Bülow P.:* A subdivision of *Staphylococcus aureus* belonging to the 83A, 84 85 6557 592 complex with special reference to antibiotic resistence. Acta path. microbiol. scand. Sect. B, 79 377–384 1971
30. *Stålenheim G., Götze O., Cooper N R., Sjöquist J & Müller-Eberhard, H J.,* Consumption of human complement components by complexes of IgG with protein A of *Staphylococcus aureus*. Immunochem. *10* 501–507 1973
31. *Winblad S & Ericson C* Sensitized sheep red cells as a reactant for *Staphylococcus aureus* protein A. Methodology and epidemiology with special reference to weakly reacting methicillin-resistant strains. Acta path. microbiol. scand. Sect. B, *81* 150–156, 1973.

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STUDIES ON POLYSACCHARIDE C OF *STAPHYLOCOCCUS EPIDERMIDIS*

1 *Isolation and Chemical Characterization*

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Johnsen, Grethe Skoge Endresen, C., Grov A. & Oeding P Studies on polysaccharide C of *Staphylococcus epidermidis*. 1 Isolation and chemical characterization. Acta path. microbiol. scand. Sect. B, 83 226–234 1975

Polysaccharide C (poly C) has been isolated from two strains of *S epidermidis* and characterized chemically The results suggest that poly C is a wall N-acetylglucosaminylglycerol teichoic acid, linked through 1.3-phosphodiester linkages. One poly C preparation contained only β-linked N-acetylglucosamine, the other traces of α linked sugar in addition. The degree of substitution of sugar in the poly C preparations from the two strains was about 50 and 25 per cent.

Key words *Staphylococcus epidermidis* polysaccharide C isolation chemical characterization.

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Polysaccharides exhibiting two strong and one weak precipitin line on double diffusion in agar were isolated from two strains of *Staphylococcus epidermidis* (3519 and 1622) by *Lornegard* & *Oeding* (31 32) One major line was identical to the *S aureus* polysaccharide (poly) Aβ (15 23) the other was supposed to be due to a group antigen present in *S epidermidis* group C (40) The polysaccharide complex was called poly AC. It was roughly characterized chemically but poly C was not isolated. *Davison et al.* (15) identified the weak precipitin line mentioned above as the *S aureus* poly Aα line (25 26) The majority of a collection of urinary strains of *S epidermidis* (erroneously classified as micrococci) produced the poly AC lines in agar (36)

Poly C of *S epidermidis* has been found to cross-react serologically with poly Aβ indicating a common antigenic determinant (19). From *S aureus* strains, isolated from pigeon and mink, a polysaccharide (poly H) cross-reacting with both poly Aβ and poly C was isolated (17 35)

In the present study poly C has been isolated and characterized chemically The antigenic properties will be reported in a subsequent paper

MATERIALS AND METHODS

Strains

S epidermidis strain 3519 (31,32) containing both poly Aβ and poly C, and strain 11997 isolated at the routine laboratory of the Institute and found to contain poly C only were used. The bacteria were grown on plates of nutrient agar 3 g

Bacto beef extract (Difco Lab USA) 10 g of ...cto peptone (Difco) 5 g of NaCl, and 15 g of ...ar made up to 1 l with deionized water

...olation of Polysaccharides

Polysaccharides were extracted from the bac...ria with a 1/15 M phosphate buffer pH 6.5 at ... C (24) without prior disruption of the bac...ria, and purified on columns of DEAE-cellulose (Eastman Org Chem., USA) and Sephadex ...-75 (Pharmacia, Sweden) The DEAE-cellulose ...as suspended in 0.02 M phosphate buffer pH 7 4 ...nd packed into columns of 2.7 × 34 cm (24) ...lution of antigenic material was performed with ...e same buffer and either (A) a gradient of NaCl, ...–0.55 M in a total volume of 1300 or 2200 ml or ...B) first elution with a gradient, 0–0.3 M NaCl ...n 700 ml, then with 900 ml of 0.3 M NaCl, and ...inally with a gradient, 0.3–0.55 M N Cl in 600 ...ml. The elution rate was 30 ml/h. The Sephadex ...-75 columns (2.5 × 96 cm) were stabilized and ...luted w th an 0 1 M Tris-HCl buffer pH 8.0 ...containing 0.5 M KCl, at a rate of 30 ml/h.

Isolation of Cell Walls

Isolation and purification of cell walls using the method of *Yoshida et al.* (43) was as described in (20) The layer just below the cell wall material on gradient centrifugation, supposed to contain membrane fragments, was also subjected to further purification on gradient centrifugation.

Analytical Methods

Hydrolysis. Samples (5–10 mg) of polysaccharide were hydrolysed in sealed tubes, flushed with nitrogen, in 0.5 ml volumes of (a) 0 1 N HCl for 2 h at 100 C (examination for nucleic acids) (b) 3 N HCl for 3 h at 100° C and ( ) 1 N NaOH for 3 h at 100° C (examination for neutral sugars, amino sugars, sugar alcohols and phosphate esters) and (d) 6 N HCl for 16 h at 105 C (examination for amino acids)

Ion-exchange col mns. Amberlite IR 120 (H) 100–230 mesh, 8 per cent cross-linked (BDH, Ltd., England) was used to isolate amino acids from acid hydrolysates. The resin, suspended in distilled water was placed in a column of 1 × 6 cm. After application of hydrolysed material (in distilled water) elution was performed with 5 × 2 ml of distilled water at a rate of 4 ml/min, then 5 × 1 ml of 7N NH OH at rate of 1 ml/min followed by 3 ml of distilled water

Fractionation of sugar alcohols, amino sugars and phosphate esters in alkaline hydrolysates was carried out on a column (1 × 6 cm) containing the anion exchange resin AG-2 10 acetate form, 200-400 mesh (Bio-Rad., USA) The elution was performed with 8 × 1 ml of distilled w ter (neutral fraction) followed by 1 ml of 6 N HCl and 4 ml of 1 N HCl (acid fraction) For larger amounts than 10 mg of polysaccharide, a column of 1 4 × 27 cm, a neutral fraction of 70 ml, and an acid fraction of 200 ml obtained by an 0–4 N HCl gradient, were used. The neutral fractions were shaken with Amberlite IR 120 (H^+) to remove Na-ions, and all fractions were then lyophilized.

Digestion with alkaline phosphatase Test samples were treated with alkaline phosphatase (Type II Sigma, USA) in 0 01 M (NH_4) CO_3, pH 9.3 for 12 h at 37 C (3 41) The enzyme was precipitated by 2 N HCl and removed by centrifugation. The acid supernatant was divided, one part being heated for 3 h at 100 C in a sealed tube. After evaporation to dryness, the resultant residues were examined by chromatography

Chromatography Circular and descending chromatography were carried out on Whatman No 1 paper with the following solvent systems

A Isopropanol 2 N HCl (65:35, v/v) (30)
B. Phenol H_2O (4 1 w/v) (38)
C. Butanol HAc:H_2O (4 1 1 v/v) (38)
D Propanol NH_3 H_2O (6.3 1 v/v) (5)
E. Ethylacetate pyridine H_2O (40 11·6, v/v) (18)

The detecting reagents employed were ninhydrin (33) with or without collidine (38) (amino acids and amino sugars) the Elson-Morgan reagent (37) (amino sugars) alkaline silver nitrate (35) (sugar ...lcohols, reducing sugars and amino sugars) sodium periodate-benzidine (13) (sugar alcohols) and the perchloric acid-ammoniummolybdate-H_2S reagent of *Hanes & Isherwood* (21) (phosphate esters)

Quantitative determination of neutral carbohydrates and sugar alcohols as trifluoroacetylated derivatives of the corresponding alditols was carried out on Perkin Elmer 900 gas chromatograph with glass columns (0.175 × 180 cm) packed with 3 per cent OV 225 (Supelco, USA) coated on Chromosorb W (80–100 mesh) (BDH) as described by *Imanari et al.* (27) and by *End ers* (in press) The carrier gas, N_2, had a flow rate of 25–35 ml/min, the injector temperature was 210 C, the manifold temperature 260 C and the temperature of the columns 70–150° C, being raised by 4 C/min. Trimethylsilylmethylglycoside derivatives of the carbohydrates were prepared using Sylon-BTZ (Supelco) and methylated fatty acids were prepared as described by *Clamp et al.* (14) and examined by gas chromatography on columns of Chromosorb W coated with 3 1 per cent SE 30 (Serva, BRD) Separation of the sugar derivatives was obtained by temperature rise of 4 C/min from 100–210° C, whereas methylated fatty acids, including myristic acid, palmitic acid,

and stearic acid (Pierce Chem. Co., USA) were chromatographed isothermically at 160 C.

Quantitative analysis of amino acids as trifluoroacetylated butyl esters (39) was also performed by gas chromatography on columns (0.2 × 200 cm) of Tabsorb (Regis Chem. Co., USA). The flow rate of the carrier gas (N_2) was 25–30 ml/min, the temperature being raised from 100–210 C at 5 C/min followed by 10 min at 210 C.

Hexosamines were determined by a modified Elson-Morgan method (28) and phosphorus as described in (44).

Reaction with concanavalin A (Con A). Con A isolated from jack-bean meal (Sigma) as described in (1) was used in a concentration of 10 mg/ml of phosphate-buffered saline, pH 7.2, the polysaccharide in 1 mg/ml. The test was carried out in 1 per cent agar, poly Ba, 1254 (32) being included as a positive control.

Serological Tests

Ring test and double diffusion in agar were performed as described earlier (24, 32) using antiserum to *S. epidermidis* 3519 and *S. aureus* Wood 46 produced in rabbits by intravenous injection of formalin-killed bacteria (34).

RESULTS

After 3 extractions the yield of crude poly C from 70 g (wet weight) of *S. epidermidis* 11997 was 560 mg. Three extractions of 90 g of 3519 bacteria yielded 650 mg crude polysaccharide (poly AβC). The bacteria still contained some poly C.

Purification on DEAE-cellulose

Fractionation of 350 mg crude poly C, 11997 on DEAE-cellulose, using elution procedure (A) is illustrated in Fig. 1. Serologically positive fractions were localized by ring test and further examined by double diffusion in agar. Poly C appeared in fraction no. 109 at an NaCl-concentration of 0.37 M, and was present in 25 fractions which also contained some UV-absorbing material. These fractions were bulked, concentrated by distillation under reduced pressure, dialyzed and refractionated on a similar column. Little or no further purification was, however, obtained on the second fractionation.

Two hundred mg crude poly AβC, 3519 were fractionated on DEAE-cellulose, using elution procedure (B). Elution of serologically active material (poly Aβ) started at 0.1 M NaCl, but the bulk of poly Aβ was eluted at 0.3 M NaCl. Poly C appeared in trace amounts after 900 ml of 0.3 M NaCl and was completely eluted with a gradient from 0.3 to 0.47 M NaCl. These fractions were concentrated, dialyzed and refractionated on a similar column, using the elution gradient A in a total volume of 2200 ml. Poly C was eluted in the range 0.34–0.45 M NaCl, whereas poly Aβ could not be detected in this range by agar gel diffusion. The optical density pattern was essentially the same as shown in Fig. 1.

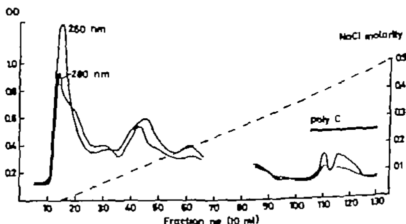


Fig. 1. Fractionation of poly C, 11997 on DEAE-cellulose. 0.07 M phosphate buffer pH 7.4 and a NaCl-gradient (– – – –).

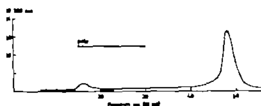


Fig. 2 Gel filtration on Sephadex G-75 of 80 mg poly C, 3519 in 0.1 M Tris-HCl pH 8.0 containing 0.5 M KCl, showing the range of poly C-positive fractions and the optical density pattern.

Gel Filtration on Sephadex G-75

Fractionation of 10 mg crude poly AβC, 3519 gave incomplete separation of the two antigens. In the first 20 ml after void volume only poly C could be detected. The next 30 ml contained both poly C and poly Aβ and the following 10 ml poly Aβ only. The gel filtration removed most of the contaminating UV-absorbing material. Purification by gel filtration of 80 mg poly C, 3519 after two DEAE-cellulose columns, is illustrated in Fig. 2. Poly C was eluted in the first 150 ml after void volume. Most of the UV-absorbing material after DEAE-cellulose fractionation appeared to be made up of low molecular substances which were eluted after 300 ml of buffer had passed the column. The yield after Sephadex filtration was 52 mg poly C, this being the final material used for analyses. Sephadex gel filtration of 60 mg poly C, 11997 after two DEAE-cellulose columns, gave 45 mg poly C, the optical density pattern being mostly the same as in Fig. 2.

The ring test titre of the polysaccharides increased from 100 to 1000 after 2 fractionations on DEAE-cellulose and 1 gel filtration. Further gel filtrations of the polysaccharides did not increase the titre. The final poly C, 11997 (5 mg/ml) showed an O.D. at 280 and 260 nm of 0.265 and 0.400, respectively. The corresponding data for poly C, 3519 were 0.085 and 0.145. No spots related to nucleic acids were detected after chromatography of hydrolysate (a) in solvent system A indicating that less than 0.1 per cent of nucleic acids was present in the polysaccharide.

Chemical Analyses

Chromatographic examination of hydrolysates (b) and (c) in solvent systems, C, D and E showed the presence of N-acetylglucosamine, glycerol, and traces of muramic acid. Using high concentration of hydrolysed poly C, 3519 trace amounts of ribitol were observed. These findings were confirmed by gas chromatography (Fig. 3). In addition to the two major peaks, representing glycerol and N-acetylglucosamine, poly C, 3519 seems to

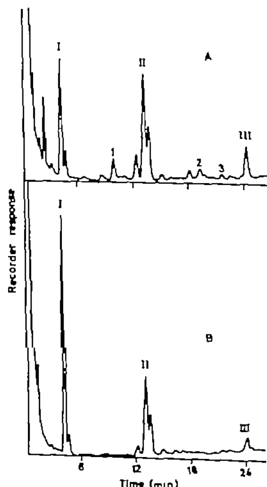


Fig. 3 Gas chromatography of trimethylsilylmethylglycoside residues. A. poly C, 3519. B. poly C, 11997. Peaks I, II and III represent glycerol, xylose (intern standard) and N-acetylglucosamine, respectively. Peaks 1, 2 and 3 in A correspond to anhydroribitol, glucose, and N-acetylgalactosamine, respectively.

contain traces of ribitol (peak 1 represents anhydroribitol) glucose and N acetylgalactosamine, all of which presumably are contaminants. *Quantitative calculations of free glycerol* in hydrolysates of poly C, 3519 and 11997 gave 3.2 and 7 7 per cent, respectively Similar results, 3 4 and 7.9 per cent, were obtained on chromatography of trifluoroacetylated *derivative of the alditol.* Quantitation of hexosamines by this method was not reproducible.

Examination of hydrolysates (b) and (c) on chromatography in solvent D using the phosphate ester reagent showed the presence of glycerol phosphate esters. Thus, only a part of the total glycerol is free after hydrolysis and the values estimated by gas chromatography are too low The conditions of hydrolysis may thus be the reason for the observed difference in the amount of glycerol in poly C 3519 and 11997

Paper chromatography of 6 N hydrolysates in solvent systems B and C revealed the presence of alanine, glycine, lysine glutamic acid, and serine. Gas chromatography showed the amounts to be 0 09 0 11 0 04 0.05 and 0 04 μmole/mg respectively In addition trace amounts of valine were observed. The type of amino acids and the molar proportions, together with the observed trace of muramic acid, indicate the presence of a small amount of mucopeptide fragments in the polysaccharides.

The colorimetric analyses of phosphorus and N-acetylglucosamine gave 6.3 and 13 1 per cent, respectively in poly C, 11997 and 4 4 and 17.2 per cent in poly C, 3519, the molar ratio N-acetylglucosamine/phosphorus thus being *0.29/1* and *0.56/1* *Fatty acids* could not be detected by gas chromatographic analysis of 2 mg polysaccharide Free alanine was not detected after incubation of polysaccharide in 1 N NH_4OH at 100°C for 5 min.

Preparative Paper Chromatography

In these studies poly Bz, 1254 (31) was included as standard glycerol teichoic acid. Polysaccharides were hydrolysed in 1 N NaOH and fractionated on AG-? ×10. Chromatography was mostly performed in solvent D the R_f-value given referring to that system. Acid fractions of both poly C preparations as well as poly Bz contained phosphate-esters with R_f-values of 0.38 and 0 15 corresponding to glycerolmono- and glyceroldiphosphate respectively (29) The *neutral fractions did not contain phosphorus* but glycerol (R_f = 0.87) and a substance

Poly C 11997 (50 mg)

Alkaline hydrolysis (1 N NaOH)
↓
Column of AG-? × 10 anion exchange resin

Neutral fraction
Glycerol (R_f = 0.87)
unknown substance (R_f = 0.58)
↓ 3 N HCl hydrolysis
glycerol
N-acetylglucosamine

Acid fraction
Glycerol monophosphates (R_f = 0.38)
Glyceroldiphosphates (R = 0.15)
↓ Alkaline phosphatase
Inorganic phosphate
glycerol
glycerolmonophosphate
phosphate esters (R_f = 0.57)
↓ 1 N NaOH hydrolysis
glycerol
glycerolmonophosphates

Fig 4 Scheme of analytical procedure

R_f = 0.58) which upon acid hydrolysis (3N HCl) was found to be composed of glycerol and glucosamine. When eluted material corresponding to glyceroldiphosphate (R_f = 0.15) was digested with alkaline phosphatase glycerol, inorganic phosphate, glycerolmonophosphate, and a substance with an R_f = 0.57 appeared on the chromatogram. This substance was shown to be a phosphate-ester which after hydrolysis in 1 N NaOH gave glycerol and glycerolmonophosphate (Fig. 4).

Double Diffusion in Agar

Poly C, 11997 gave a weak line against Con A, whereas no line was observed with poly C, 3519.

The cell wall preparation of strain 3519 (1 mg/ml) gave lines against anti 3519 serum fusing completely with poly C and poly Aβ. In contrast, the material supposed to be membrane fragments showed no precipitin reaction with the serum.

DISCUSSION

Poly C was easily extractable from *S. epidermidis* strain 11997, a relatively good yield being obtained. Strain 3519, previously utilized for isolation of poly AβC (31), gave approximately the same yield of polysaccharide, this being a mixture of poly Aβ and C.

Ion-exchange chromatography on DEAE-cellulose increased the purity of poly C considerably as judged from UV-absorption and ring test titre. The latter increased by a factor of 4–5. The two antigens of crude poly 3519 (AβC) were separated on DEAE-cellulose. In the conditions applied, poly C was more strongly bound to the resin than poly Aβ indicating that the former had a stronger negative charge.

Gel filtration on Sephadex G-75 gave an additional reduction of UV-absorbing material and an additional two-fold increase of the ring test titre. The partial separation of poly Aβ and poly C by gel filtration may indicate a higher molecular weight of poly C than of poly Aβ. A combination of ion-exchange chromatography and gel filtration seemed to give satisfactorily pure preparations. The UV-absorbing material was negligible and without influence on the chemical analyses, and neither lipids nor proteins were present in detectable amounts.

The presence of poly C in the cell wall preparations in contrast to the membrane-containing fraction, and the absence of lipids in poly C, show that poly C is a cell wall material without relationship to membrane teichoic acids (42).

The components released by acid hydrolysis, especially glycerol diphosphate, indicated that poly C is a glycerol teichoic acid with the glycerol linked through phosphodiester linkages. An attempt was made to resolve the question of whether the phosphate linkages occupy the 1.3 positions or the 1.2 positions by preparative paper chromatography including alkaline and enzymatic hydrolyses, following the principle that phosphodiesters are hydrolysed with alkali only if they contain at least one hydroxyl group on a carbon atom adjacent to that which bears the phosphodiester grouping (10, 11). Alkaline hydrolysis of a 1.2-polymer of glycerophosphate units may proceed in two ways (29), giving either glycerol and its mono- and diphosphates or glycerol monophosphates only. Alkaline hydrolysis of a 1.3-linked structure gives glycerol and glycerol mono- and diphosphates. In addition 1.3-polymers may in contrast to 1.2 polymers, give diglycerol triphosphate (29), which can be detected by enzymatic release of the terminal phosphate groups followed by alkaline hydrolysis.

In the present study the material corresponding to diphosphates (R_f = 0.15) was digestible with alkaline phosphatase giving a substance with an R_f-value of 0.57. This substance gave only glycerol and glycerol monophosphates upon further hydrolysis in alkali, indicating that the substance (R_f = 0.57) was a diglycerol phosphate most probably formed by enzymatic hydrolysis of diglycerol triphosphate. Thus, poly C most likely contains a 1.3-phosphodiester structure.

Preparative paper chromatography also revealed components in which N-acetylglucosamine was linked directly to glycerol, indicating a (1→2) glucosaminylglycerol teichoic acid. A 1,3-linked glycerophosphate polymer in which all the 2 positions of glycerol are substituted should be stable to alkali (10 11) Therefore, the observed glycerol-glucosamine component present in alkaline hydrolysates indicated an incomplete substitution of the polymer The degree of substitution may vary within chains and in preparations from different strains (12, 2) The molar ratios of N-acetylglucosamine and phosphorus in poly C from strains 11997 and 3519 were 0 23/1 and 0 46/1 respectively after correction for N-acetylglucosamine derived from mucopeptide fragments (0 1 μmole/mg based on the content of amino acids) According to these figures the substitution with N-acetylglucosamine in poly C, 3519 is about twice that of poly C, 11997 approximately 50 and 25 per cent, respectively Whether D-alanyl groups are attached in ester linkages to some of the glycerol residues that are not glycosylated (6) was not revealed, but the total amount of alanine present, as compared to the other amino acids, and the absence of any release of alanine after weak alkaline hydrolysis argue that possibility (22)

Concanavalin A has been shown to interact with carbohydrates containing nonreducing α D-glucopyranosyl or α D-mannopyranosyl substituents in which hydroxyl groups at positions 3, 4 and 6 are unsubstituted. The hydroxyl group at position 2 can be substituted or replaced by an acetamido group without loss of affinity for Con A (4 16) In the present experiments Con A gave a strong precipitin line with poly Ba (α-glucosylglycerol teichoic acid) (32) and a weak line against poly C, 11997 while no line was observed either with poly C, 3519 or with poly Aβ This indicates that poly C, 11997 in contrast to poly C, 3519 contains small amounts of α linked N-acetylglucosamine. The ratio α/β linkages in a teichoic acid may vary with the cultivation conditions (7) and has been found to decrease with the time of cultivation (8 9 15) Strain 11997 containing both anomers of N-acetylglucosamine, has been cultivated for a relatively short time, in contrast to strain 3519

REFERENCES

1 *Agrawal B B. I & Goldstein I J* Specific binding of concanavalin A to cross-linked dextran gels. Biochem. J 96 23c, 1965.
2. *Archibald A R., Baddiley J & Heptinstall, S* The distribution of the glucosyl substituents along the chain of the teichoic acid in walls of *Lactobacillus buchneri* N.C.I.B. 8007 Biochem. J *111* 245–246, 1969
3 *Archibald A R., Baddiley J & Shaukat G A* The glycerol teichoic acid from walls of *Staphylococcus epidermidis* I 2. Biochem. J *110* 583–588, 1968.
4 *Archibald A R. & Coapes, H E.* The interaction of concanavalin A with teichoic acids and bacterial walls. Biochem J *123* 665–667, 1971
5. *Armstrong, J J Baddiley J., Buchanan, J G Carss B & Greenberg, G R.* Isolation and structure of ribitol phosphate derivatives (teichoic acids) from bacterial cell walls. J chem. Soc. 4344–4354 1958.
6. *Baddiley J* Structure biosynthesis and function of teichoic acids. Account. chem. Res. 3 98–105 1970.
7 *Baddiley J., Buchanan J G., Hardy F E Martin R O RajBhandary U L & Sanderson A R* The structure of the ribitol teichoic acid of *Staphylococcus aureus* H Biochim. biophys. Acta (Amst) 52 406 407 1961
8. *Baddiley J Buchanan J G., Martin R. O. & RajBhandary U L.* Teichoic acid from the walls of *Staphylococcus aureus* H. 2. Location of phosphate and alanine residues Biochem. J 85 49–56, 1962.
9 *Baddiley J Buchanan J G RajBhandary U L. & Sanderson A. R.* Teichoic acid from the walls of *Staphylococcus aureus* H. Structure of the N-acetylglucosaminylribitol residues. Biochem J 82 439–448, 1962.
10 *Brown D M., Magrath D I Neilson A H & Todd A R* Hydrolysis of esters of monoribonucleotides. Nature (Lond) 177 1124 1125 1956.
11 *Brown D M & Todd A R.* Nucleotides. Part X. Some observations on the structure and chemical behaviour of the nucleic acids. J chem. Soc. 52–58 1952.
12. *Chin T Burger M M & Glaser L.* Synthesis of teichoic acids VI The formation of

multiple wall polymers in *Bacillu subtili* W-23 Arch. Biochem. Biophys. *116* 358-367 1966.

13. *Cifonelli J A. & Smith F.*, Detection of glycosides and other carbohydrate compounds on paper chromatograms. Analyt. Chem. *26* 1132-1134 1954

14 *Clamp J R Dawson G & H ugh L*. The simultaneous estimation of 6-deoxy L-galactose (L-fucose) O-mannose D-galactose 2 acetamido-2-deoxy-D-glucose (N-acetyl-D-glucosamine) and N-acetylneuraminic acid (sialic acid) in glycopeptides and glycoproteins. Biochim. biophys. Acta (Amst) *148* 342-349 1967

15 *Davison A L*., *Baddiley J Hofstad T*., *Losnegard N & Oeding P*., Teichoic acids in the walls of staphylococci. Serological investigations on teichoic acids from the walls of staphylococci. Nature (Lond.) *202* 872-874 1964

16. *Doyle R. J & Birdsell D C* Interaction of concanavalin A with the cell wall of *Bacillus subtilis*. J Bact. *109* 652-658, 1972.

17 *End esen C G ov A & O ding, P*., Immunochemical studies of three cell wall polysaccharides from animal coagulase-positive staphylococci Acta path. microbiol scand. Sect B, *82* 382-386, 1974

18. *Fischer F G & Dörfel H* Die quantitative Bestimmung reduzierender Zucker auf Papierchromatogrammen. Z physiol Chem. *297* 164-178, 1954

19 *Gro A & Helgeland S M* Immunochemical characterization of *St phylococcus pider midis* and *Micrococcu* cell walls Acta path. microbiol scand. Sect B, 79 812-818 1971

20 *Grov A & R de S* Immunochemical characterization of *Staphylococcus aureus* cell walls Acta path. microbiol scand. *71* 409-416 1967

21 *Han C S & I kerwood F A* Separation of the phosphoric esters on the filter paper hromatograms. Nature (Lond) *164* 1107-1112, 1949

22 *H ken G* Immunochemical studies on polysaccharide A of *St phylococcu ureu* 7 On the chemical basis of the serological reac ti ty and the nature of the extraction process Acta path microbiol s d 53 465 474 1962

23 *Haukes C Ellw od D C Baddiley J & Oed ng P* Ser lo c l ross-reactivity between polysac h ... *St phylococ* ... bose acid of ... Amst 57 4 ... h m biophys Acta

4 *H Le C J* ... Extraction ... ternal f om ... microbiol ... *O d g P* ... ntigenic ... Acta path.

25 *H fstad T*., Studies on the antigenic structure of the 80/81 complex of *Staphylococ us au eus*. 3 Purification and chemical characterization of a major polysaccharide precipitinogen. Acta path. microbiol. scand. *63* 59-71 1965

26. *Hofstad T* Studies on the antigenic structure of the 80/81 complex of *Staphylococcus aureus* 4 Antigenic properties and chemical basis of serological reactivity of a purified polysaccharide. Acta path. microbiol. scand. *63* 422-434 1965.

27 *Imanari, T*., *A akawa Y & Tamura Z*. Gas chromatographic analysis of aldoses. Chem. pharm. Bull. *17* 1967-1969 1969

28. *Johnson A. R.* Improved method of hexosamine determination. Analyt. Biochem. *44* 628-635 1971

29 *Kelemen M I & Baddiley J*. Structure of the intracellular glycerol teichoic acid from *Lactobacillu asei* A.T.C.C. 7469 Biochem. J *80* 246-254 1961

30 *Linskens H F* Papierchromatographie in der Botanik. Springer Verlag Berlin 1955 p. 164

31 *Lo negard N & Oeding, P* Immunochemical studies on polysaccharides from *Staphylococcu pidermidis* I Isolation and chemical characterization. Acta path. microbiol. scand. *58* 482-492, 1963

32. *Losnegard N & Oeding, P*., Immunochemical studies on polysaccharides from *Staphylococcu pidermidis*. 2. Antigenic properties. Acta path. microbiol. scand. *58* 493-500, 1963

33 *Merck E*., Chromatographie unter besonderer Berücksichtigung der Papierchromatographie E. Merck AG Darmstadt.

34 *O ding P* Agglutinability of pyogenic staphylococci at various conditions. Acta path. microbiol. scand. *41* 310-324 1957

35 *O ding P* Wall teichoic acids in animal *Staphylococcus aureus* strains determined by precipitation. Acta path. microbiol. scand. Sect. B, *81* 327-336 1973.

36 *Oeding P & Haukelgren I L*. Antigenic studies of genus *Micrococ us*. 2. Double diffusion in agar gel with particular emphasis on teichoic acids. Acta path. microbiol. scand. Sect. B, *80* 265-269 1972

37 *Partridge S M* Filter paper partition chromatography of sugars I General description and application to the qualitath analysis of sugars in apple juice egg white and foetal blood of sheep Biochem. J *42* 238-250 1948.

38 *Pataki G* Dünnschicht Chromatographie in der Aminosäure- und Peptid-chemie Walter de Gruyter & Co., Berlin 1966, p 79

39 *Roach D & Gehrk C H* The gas-liquid

chromatography of amino acids. J Chromatogr *43* 303–310 1969
40. *Thompson R & Khorazo D* Correlated antigenic and biochemical properties of staphylococci. J Bact. *34* 69–79 1937
41 *Watson M J & Baddiley J* The action of nitrous acid on C-teichoic acid (C-substance) from the walls of *Diplococcus pneumoniae* Biochem. J *137* 399–404 1974
42. *Wicken A J & Knox K W* Studies on the group F antigen of lactobacilli: isolation of a teichoic acid-lipid complex from *Lactobacillus fermenti* NCTC 6991 J gen. Microbiol. *60* 293–301 1970.
43 *Yoshida A., Heden C-G Cedergren B & Edebo L.* A method for the preparation of undigested bacterial cell walls J biochem microbiol. Technol. Engng *III* 151–159, 1961
44 *Youngburg G E. & Youngburg, M V* Phosphorus metabolism. J Lab. clin. Med. *16* 158–166, 1930.

Acta path. microbiol scand. Sect. B 83 235–239 1975

STUDIES ON POLYSACCHARIDE C OF *STAPHYLOCOCCUS EPIDERMIDIS*

2 *Antigenic Properties*

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Johnsen, Grethe Skoge Grov A. & Oeding, P Studies on polysaccharide C of *Staphylococcus epidermidis*. 2 Antigenic properties. Acta path. microbiol. scand Sect. B, 83 235–239 1975.

The antigenic properties of polysaccharide C (poly C) of *S epidermidis* strongly support the analytical indications that it is an N-acetylglucosaminylglycerol teichoic acid with 1.3-phosphodiester linkages. The major antigenic determinant is N-acetylglucosamine, predominantly present in the β-configuration Also anti-glycerophosphate antibodies are produced, apparently dependent on the degree of glycosylation. Purified poly C was unable to sensitize either normal or tanned sheep erythrocytes for agglutination in antisera.

Key words *Staphylococcus epidermidis* polysaccharide C antigenic properties

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In a previous paper (9) polysaccharide C (poly C) was isolated from two strains of *Staphylococcus epidermidis* and characterized chemically as an N-acetylglucosaminylglycerol teichoic acid linked through 1.3-phosphodiester linkages. Analysis of the two preparations indicated variations both in degree of sugar substitution and in the configuration of the sugar the β-linkage being predominant.

The present paper reports the results of serological examinations of the isolated polysaccharides and an attempt to characterize the antigenic determinants.

MATERIALS AND METHODS

Antigens

Crude and purified polysaccharide C, 11997 and 3519 were those described previously (9) Poly Aβ, *S aureus* Wood 46 (6) poly Ba *S epidermidis* 1234 (11) and poly H isolated from a pigeon *S aureus* strain (1) were included as references.

Sera

Antisera to *S aureus* strains Wood 46 and H and to *S epidermidis* 1234 and 3519 were raised in New Zealand white rabbits of the Institute's breed by intravenous injections of formalin-killed bacteria (13) Following this procedure with strain *S epidermidis* 11997 no poly C antibodies were detected in the sera. The concentration of bacteria in the injection doses (0.5 ml) was varied from 5 × 10⁹ cells/ml to 50 × 10⁹ cells/ml, and living cells as well as cells killed by formalin or by heat were tested without success. Neither did brown rabbits of another breed, nor guinea pigs and mice given number of subcutaneous and intraperitoneal injections of bacteria in Freund's incomplete adjuvant (Difco Lab., USA) give response to poly C. Five passages of the strain in guinea pigs did not seem to increase the immunogenicity of the bacteria. However after a series of intramuscular injections, 1 2 weeks part, with doses (0.5 ml) ranging from 10 × 10⁹ cells/ml to 40 × 10⁹ cells/ml in Freund' incomplete adjuvant, the sera gav

precipitation with poly C on diffusion in agar. Sera showing weak reactivity against poly C in agar diffusion were concentrated by lyophilization followed by dialysis and adjustment of the volume with saline.

Absorption of Sera

Absorption was either performed with whole bacteria, 2 × 1.5 g bacteria (centrifuged at 10 000 × g for 20 min) per ml of serum, or with purified polysaccharide, 1 mg per ml of serum. The suspensions of bacteria and solutions of polysaccharide in serum were thoroughly mixed and incubated at 37 C for 2 h, then at 4 C overnight followed by centrifugation.

Serological Tests

Ring test and double diffusion in agar were carried out as in (7) and (11) respectively the agar plates being incubated at 4 C. Poly Aβ and poly Bα were used at a concentration of 0.05 mg/ml, poly H and poly C, 3519 and 11997 at 0.1 mg/ml. Indirect haemagglutination, using both normal and tanned sheep erythrocytes, was performed as described in (14) and immunoelectrophoresis was carried out with an LKB 6800 apparatus in 1 per cent agar Noble in sodium veronal buffer pH 8.7 I 0.05 for 1 h at 10 V/cm.

Periodate Oxidation

Samples of polysaccharides (1 mg) were treated with 0.1 and 0.5 per cent solutions (1 ml) of sodium metaperiodate at room temperature in the dark for 20 h. After dialysis against distilled water for 10 h, the solutions were tested for serological activity by double diffusion in agar.

RESULTS

Double Diffusion in Agar

Suspensions of 3519 bacteria as well as crude poly AβC, 3519 gave 2 lines against homologous antisera, the line next to the serum well being identical with the poly Aβ line (Fig. 1 A). The other line was due to poly C, this being the only line produced by the purified poly C preparations (Fig. 1 B). Purified poly C and poly Aβ produced lines of partial identity against antiserum to 3519 bacteria the poly C line spurring over that of poly Aβ (Fig. 2). Diffusion against anti Wood 46 sera gave however a reaction of complete identity between the two polysaccharides.



Fig. 1. Double diffusion in agar. A shows poly Aβ and poly C precipitation lines between *S. epidermidis* 3519 (1) and homologous antiserum (2). The poly Aβ line closest to the serum well, fuses completely with the line between poly Aβ (4) and anti-*S. aureus* Wood 46 serum (3). B shows that *S. epidermidis* 3519 (1) and crude poly AβC (2) give the same lines against anti-3519 serum (5). Purified poly C 3519 (3) and 11997 (4) give the poly C line only.



Fig. 2. Double diffusion in agar showing a reaction of partial identity between poly C (1) and poly Aβ (3) against anti-3519 serum (2).

All antisera to 3519 bacteria and all antisera to 11997 bacteria except one (see below) gave an identical line against poly C. Further all purified poly C preparations showed serological identity with poly H in agar diffusion both with anti-3519, anti-11997 and anti-H_1 sera. One of the antisera to strain 11997 (Ö 152) produced no true poly C line but a weak atypical line against the homologous strain and isolated poly C (Fig. 3). The line showed partial identity to the poly C line, but could not be produced with poly C, 3519 or poly Aβ. These antigens were also negative on ring test against serum Ö 152.

After oxidation of crude poly AβC, 3519 in 0.1 per cent periodate the poly Aβ line disappeared, whereas the poly C line seemed



Fig. 3. Double diffusion in agar showing the specific line between anti-*S. epidermidis* 11997 Ö 152 (1) and poly C, 11997 (3). Anti-3519 serum in well (2).

unaffected. When 0.3 per cent periodate was used, both lines disappeared. Periodate oxidation (0.3 per cent) of poly C, 11997 destroyed the poly C-line determinant, whereas the line with serum Ö 152 was still produced, giving a reaction of identity with that formed by untreated polysaccharide. Serum Ö 152 was the only one to react with periodate-oxidized polysaccharide.

Absorptions

Antisera to strains 3519 1254 and Wood 46 were absorbed with homologous bacteria and found to be exhausted for precipitins as shown by double diffusion in agar Cross-absorptions revealed that 1254 bacteria did not change the precipitating ability of anti 3519 and anti-Wood 46 sera as tested against poly C and poly Aβ On the other hand, both these sera, in contrast to anti-1254 serum, were exhausted upon absorption with 3519 Wood 46 or 11997 bacteria.

Two antisera to strain 11997 Ö 150 and Ö 152, were both concentrated to four fold strength, absorbed with purified Aβ or poly C, 11997 and then tested for residual precipitins. Absorption with poly C removed all precipitins from both sera, whereas poly Aβ removed only antibodies responsible for the poly C line. The precipitins of Ö 152, forming the weak line against poly C, 11997 were unaffected by poly Aβ

Indirect Haemagglutination

Normal and tanned sheep erythrocytes were sensitized with both crude and purified polysaccharide preparations and tested for agglutination in twofold dilutions of homologous and heterologous antisera. No strain difference was observed. The haemagglutination titres of anti-3519 and anti-11997 sera were 1/20 and 1/80 respectively of both normal and tanned cells sensitized with crude materials. Normal and tanned cells treated with purified preparations did not agglutinate in any of the sera.

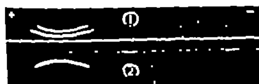


Fig 4 Immunoelectrophoresis of poly C + poly Aβ (1) and poly C (2) both antigens at concentration of 1 mg/ml Anti-3519 serum in the trough.

Immunoelectrophoresis

No difference was observed on immunoelectrophoresis of poly C from the two strains, and all purified poly C preparations apparently had the same electrophoretic mobility as that of poly Aβ (Fig 4) Two lines were produced by a mixture of poly Aβ and poly C with the poly Aβ line closest to the serum trough. Poly C seemed to be a little more homogeneous in charge than poly Aβ The charge difference indicated by the DEAE-cellulose fractionation (9) was not observable in the conditions used in immunoelectrophoresis. Poly C and poly H showed identity both in electrophoretic mobility and in immunodiffusion (Fig 5) The same pattern was revealed both with anti-3519 and anti-H serum.



Fig 5 Immunoelectrophoresis of poly C + poly H (1) and poly H (2) both antigens at a concentration of 1 mg/ml Anti-3519 serum in the trough.

DISCUSSION

The lack of antibody response to poly C, 11997 on injection of whole cells according to usual administration routes is most probably due to several factors. It has previously been found difficult to produce potent antisera against similar polysaccharides. Such substances, being non-immunogenic in purified state apparently have to be bound to other cell structures (3) Passage in guinea

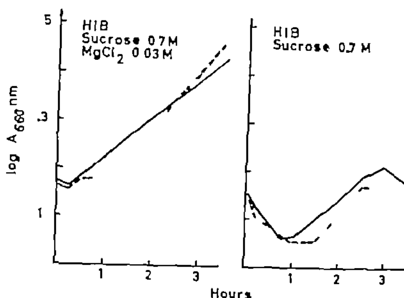


Fig 1 Effects of the transfer of a *N meningitidis* culture into HIB made hypertonic with sucrose Comparison of the *cp* variant (solid lines) and *cp* variant (dotted lines) A HIB culture in the exponential phase was harvested in the centrifuge and resuspended in the same volume of HIB supplemented with 0 7 M sucrose and $MgCl_2$ as indicated.

RESULTS

Growth after exposure to high tonicity After resuspension of log phase cells from the *N meningitidis* competence variants in complete media made hypertonic with sucrose there was always a pronounced lag before the growth was resumed. This lag which was always longer in the *cp* variant than in the *cp*+ one, could be substantially shortened by the addition of $MgCl_2$ (Table 1) During the lag period absorbancy regularly decreased, but the decline was reduced by the addition of $MgCl_2$ (Fig. 1) The addition of $CaCl_2$ also reduced the decline in absorbancy but it had very little or no influence on the lag

Hypertonic conditions also slowed down the growth rate in meningococcal cultures, more in the *cp*+ than in the *cp* variant (Table 1) The addition of MgCl did not seem to have any significant effect on the generation time under these conditions. However in the absence of $MgCl_2$ growth stopped very soon and was followed by lysis of the culture (Fig 1) Cells that were no longer in the exponential phase did not initiate growth if resuspended in the HIB-sucrose media and the cultures underwent lysis.

Phase contrast microscopy immediately after the exposure to increased osmotic pressure revealed changes from the normal appearance. Most of the cells appeared comparatively small with very intense contrast. Often the cells showed structures near the surface, comparable to the plasmolysis vacuoles observed in other bacteria (1) Many cells, however did not show typical vacuoles, but at the same time did not appear normal, being particularly small and irregular in shape. Collapsing cell walls may have been the cause of the abnormal appearance Phase contrast microscopy at a later stage after the resumption of growth gave a picture virtually indistinguishable from that of the original culture.

Autolysis in suspensions of meningococci. Fig 2 shows that intact *N meningitidis* cells autolysed if suspended in various solutes. Lysis was comparatively slow over a rather broad range of concentrations between 0.1 M and 0.3 M (from approximately 5.5 atm osmotic pressure to around 13 atm.) but it was generally slightly faster in the *cp* variant

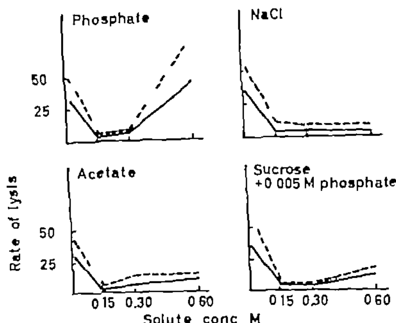


Fig 2 Effect of solute concentration on the rate of autolysis. Exponential phase cultures were harvested in the centrifuge washed once with 0.15 M of the solute under study and resuspended in the solute concentration shown. Autolysis was measured at 20 C during 2 hours and calculated as described in Materials and Methods. Solid lines *cp* variant dotted lines *cp* variant.

than in the *cp* one. If measured with phosphate buffer the rate of lysis would be nearly constant over a fairly broad pH range between pH 6 and pH 7.5

It appears from Fig 2 that the lysis in all the solutes recorded increased upon exposure to hypotonic conditions. The rate of lysis after exposure to 0 015 M was virtually the same whether measured at 0.15 M or at 0.015 M (Fig. 3) But if the suspensions were exposed to 0.015 M an almost instantaneous lysis of a large part of the cells took place (Fig 4 Curve A) whereas the successive lysis occurred at the rate recorded in Fig. 2 during the following two hours. The immediate lysis was always more pronounced in the *p* variant (80–90 per cent) than in the *cp*⁺ (60–70 per cent) Curve B in Fig 4 shows that the increase in the rate of lysis subsequent to the exposure was also more pronounced in the *cp* variant.

Fig 2 shows that the rate of lysis was very much enhanced if measured in the presence of 0.6 M phosphate. But it is also seen that the rate of lysis would not be increased if 0.6 M of NaCl was used and very little, if any if 0.6 M sucrose or 0 6 M acetate was used. This could obviously be due either to an inhibition of autolysis, or to osmotic stabilization of the membranes. Stabilization is usually achieved by adding appropriate concentrations of non-penetrable solutes (4) Fig 5 shows the time-dependent activation of autolysis, and the development of osmotic fragility in suspensions of *N meningitidis* during exposure to 0 6 M of various solutes. In fact the results indicate that very little lysis takes place in 0.6 M NaCl, whereas 0 6 M acetate as well as 0.6 M sucrose acts as osmotic stabilizer The solutes obviously activate the autolytic process, although the activation caused by 0.6 M NaCl is very feeble. In addition, phosphate seems to cause lysis of the cell membrane since it does not stabilize the suspension if added in appropriate concentration.

The rate of autolysis was inhibited by divalent cations (Table 2) $CaCl_2$ was particularly

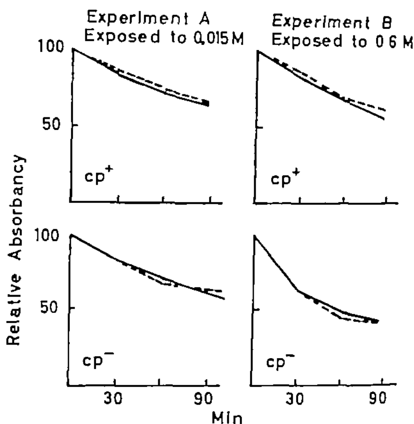


Fig. 3 Changes in absorbancy after exposure to 0.015 M (Expt. A) and 0.6 M (Expt. B) phosphate for 30 min. Solid lines measured with 0.15 M phosphate broken lines measured with 0.015 M (Expt. A) or 0.6 M (Expt. B) phosphate.

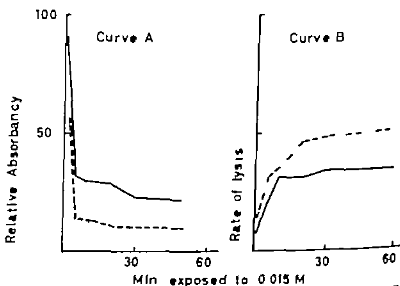


Fig. 4 *Curve A* Initial lysis of cells after exposure to 0.015 M phosphate. *Curve B* Influence of exposure to 0.015 M phosphate on the rate of lysis. All measurements performed after readjustment to 0.15 M. Solid lines *cp* variant dotted lines *cp* variant.

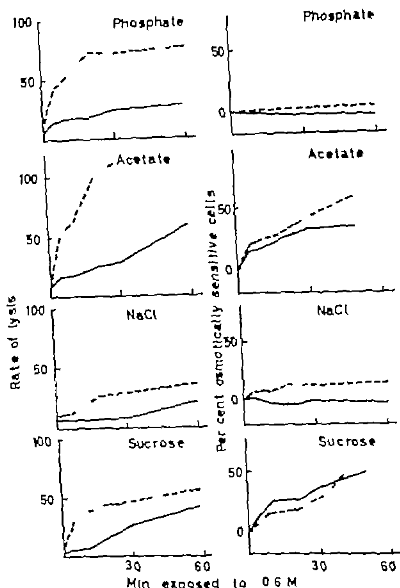


Fig. 5. Activation of autolysis and development of osmotic fragility during exposure to 0.6 M of various solutes. Exponential phase cells were harvested in the centrifuge, washed once with 0.15 M of the solute to be used in the experiment and resuspended in 0.6 M of the solute. (The cells to be used in the sucrose assay were washed with 0.15 M acetate.) At the time shown, the concentration was readjusted to 0.15 M. Rate of lysis and development of osmotic fragility was measured as described in Materials and Methods. Comparison of *sp*+ cells (solid lines) and *sp* cells (dotted lines).

effective, resulting in more than 40 per cent inhibition in the concentration 0.005 M. In contrast, $CaCl_2$ and $MgCl_2$ differed with respect to their effects on lysis in the presence of 0.6 M acetate or 0.6 M sucrose. Table 3 shows that $CaCl_2$ abolished the stabilizing effect of 0.6 M acetate while $MgCl_2$ increased the stability. Similar results were obtained if sucrose was used as the main stabilizer. Thus, it seems that $CaCl_2$ activates the lysis of the cytoplasmic membranes whereas $MgCl_2$ counteracts such lysis.

TABLE 2 *Inhibition of Autolysis Rate by $MgCl_2$ and $CaCl_2$*

Competence variant	Per cent inhibition of autolysis rate			
	$MgCl_2$		$CaCl_2$	
	0.05 M	0.005 M	0.05 M	0.005 M
cp	58	16	72	48
cp	46	15	73	43

* = Measured in the presence of 0 15 M acetate.
Cells from the exponential phase were exposed to 0.015 M acetate for 30 min to activate autolysis. Inhibition is given as the per cent reduction of the rate with 0.15 M acetate only

TABLE 3 *Abrogation of the Stabilizing Effect of 0.6 M Acetate by CaCl*

Competence variant	Rate of lysis					
		$MgCl_2$			CaCl	
	None	0.05 M	0.005 M	None	0.05 M	0 005 M
cp	4.8	2.3	3.8	7 1	16.0 (57)	26.3 (50)
cp	5.4	3.4	4 6	13.3	17.3 (64)	33 6 (62)

* = Measured in the presence of 0.6 M acetate. Numbers in brackets represent calculations based upon the inhibition of the rate of autolysis observed with 0.15 M acetate (Table 2)
Cells from the exponential phase were washed once with 0.15 M acetate and resuspended in 0 6 M acetate plus $MgCl_2$ or $CaCl_2$ as indicated. Lysis was measured during two hours.

TABLE 4 *Comparison of Rate of Autolysis in Exponential Phase Cells and Stationary Phase Cells*

Competence variant	Rate of autolysis					
	Exponential phase			Stationary phase		
	Kept at 0 15 M	Exposed to 0.015 M 30 min	Exposed to 0 6 M 30 min	Kept at 0.15 M	Exposed to 0 015 M 30 min	Exposed to 0 6 M 30 min
cp	5.0	37 1	28.9	8.6	5.0	9 0
p	7.8	54 7	118.5	10.1	7 3	17 8

Experiment with acetate. Rat of autolysis was measured with 0 15 M acetate as described in Materials and Methods.

Autolysis also depended on the age of the cells (9 10) Table 4 shows that the rate of lysis is nearly the same in log phase cells and in stationary phase cells provided the cells are kept scrupulously around 0 15 M However the activation of autolysis is practically absent in the stationary phase cells. The stationary phase cells did not develop osmotic fragility if incubated in 0 6 M acetate or 0 6 M sucrose in the way it has been demonstrated in Fig 5 in the case of exponential phase cells.

DISCUSSION

As expected from previous observations (6) exposure of *N. meningitidis* cultures to hypertonic media resulted in changes corresponding to plasmolysis. But deplasmolysis obviously takes place in the presence of nutrients

and ions as previously observed in *N meningitidis* (6) as well as in other bacteria (12). The lag found after resuspension of the cells in the HIB-sucrose medium is probably due to the harmful effects of plasmolysis and may represent a recuperation period. It has previously been shown that incubation in complete medium after exposure to hypertonic solutions may "repair" the damage (6). It is also noted that the lag is regularly longer in the *cp* variant than in the *cp*+ one. This may be a reflex of the relative resistance of the *cp* variant against damage due to osmotic stress (6). The growth rate in both competence variants was much reduced when growing in hypertonic media, but far more in the *cp* than in the *cp* variant. Differences in the regulation of cell division in the two variants have previously been noted (5) and there are also differences in the growth in stationary fluid cultures (2).

During the lag period absorbancy declined, indicating lysis of the cells. The subsequent experiments indeed showed that exposure of *N meningitidis* cells to conditions conductive to plasmolysis as well as plasmoptysis (6) resulted in a pronounced activation of autolysis. In contrast, cells that had been maintained at an osmotic pressure around 7 atmospheres had a very slow rate of autolysis. Exposure to low tonicity also resulted in an almost instantaneous lysis of a large part of the cells. This seems to correspond well with the very rapid killing previously observed (6) and may represent an osmotic explosion due to uptake of water.

The enzymes that cause solubilization of the peptidoglycan network of the bacterial cell wall represent integral parts of the wall structure (13, 15). Normal growth must obviously involve a harmonious balance between the lytic enzymes and synthetic ones. But the balance may apparently be disturbed in several ways. Well-known is the effect of inhibitors of cell wall synthesis such as penicillin. It now appears that plasmolysis or plasmoptysis may regularly activate autolysis in *N meningitidis*. It has previously been suggested that increased osmotic pressure activates autolysis in *Staphylococcus aureus* (9). It is also of interest that most active autolysis in intact bacteria has usually been observed after washing of the cells in distilled water (4, 9, 10). As found in other systems (9, 10) maximal autolytic activity in *N meningitidis* could be demonstrated during the exponential growth phase. But most of the activity in log phase cells is latent and requires activation, whereas stationary phase cells seem to have little or no latent activity.

As observed in several other bacteria (4, 9, 10) osmotic fragility developed in *N meningitidis* during autolysis when the suspensions were osmotically stabilized. Suitable stabilizers were sucrose and acetate. Phosphate was unsuitable since it seemed to interfere with the capability of the cell membrane to maintain an osmotic barrier. NaCl could not be used because very little lysis took place in the presence of high concentrations (0.5–0.7 M). One reason could be that high concentrations of NaCl inhibit autolysis, but this interpretation seems to disagree with the findings in *Streptococcus pneumoniae* in which cell wall autolysis is apparently stimulated by NaCl (3). It may also be, however, that NaCl in high concentration inhibits the activation of autolysis generally caused by plasmolysis.

In agreement with the findings in many other bacteria (4, 10) divalent cations had pronounced influence on cell lysis. $MgCl_2$ as well as $CaCl_2$ inhibited the rate of autolysis. As expected, the presence of $MgCl_2$ also resulted in increased osmotic stability in both 0.6 M sucrose and 0.6 M acetate. But $CaCl_2$, even in low concentration and in spite of its inhibition of the rate of autolysis, completely abolished the stabilizing effect of sucrose and acetate. A tentative interpretation is that Ca^{++} activates membrane lysis. It is of interest that Ca^{++} in the same concentration is required for transformation of *N meningitidis* (7). Ca^{++} is also of importance in several other transformation systems, and in transfection (7, 8, 14).

The findings discussed in this paper support the previous notion that plasmolysis and

plasmoptysis cause far more damage in *cp* cells than in the *cp* cells. In addition to the damage to the membrane functions previously observed (6) the cell wall complex seems to be disturbed so that autolysis is activated

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REFERENCES

1. *Birdsell, D C & Cota-Robles E. H* Production and ultrastructure of lysozyme and ethylenediaminetetraacetatelysozyme spheroplasts of *Escherichia coli*. J Bact *93* 427–437 1967
2. *Frøholm L. O Jyssum K. & Bøvre K.* Electron microscopical and cultural features of *Neisseria meningitidis* competence variants. Acta path. microbiol. scand. Sect. B, *81* 525–537 1973
3. *Howard L. V & Gooder H* Specificity of the autolysin of *Streptoc ccus* (*Diplococcus*) *pneumoniae* J Bact. *117* 796–804 1974
4. *Joseph R & Shockman G D* Autolytic formation of protoplasts (autoplasts) of *St ptococcus faecalis* 9790 Release of cell wall, autolysin, and formation of stable autoplasts. J Bact. *118* 735–746 1974
5. *Jyssum K* Cell division after inhibition of DNA and protein synthesis in *Neisseria meningitidis* 2. Residual division and increase in absorbancy after addition of hydroxylamine and chloramphenicol. Acta path. microbiol. scand. Sect. B, *81* 127 137 1973.
6. *Jyssum K.*, Influence of osmotic pressure on transformable and non transformable variants of *Neisseria meningitidis* I Survival and turbidity changes. Acta path. microbiol. scand Sect. B, *82* 484–494 1974
7. *Lie S.*, Studies on the phenotypic expression of competence in *Neisseria meningitidis*. Acta path. microbiol scand. *64* 119–129 1965.
8. *Mandel, M & Higa, A* Calcium-dependent bacteriophage DNA infection. J molec. Biol *53* 159–162, 1970
9. *Mitchell, P & Moyle J* Autolytic release and osmotic properties of "protoplasts" from *Staphylococcus aureus*. J gen. Microbiol *16* 184–194 1957
10. *Mohan, R R. Kronish, D P., Pianotti, R S Epstein R L. & Schwartz, B. S* Autolytic mechanism for spheroplast formation in *Bacillus cereus* and *Escherichia coli*. J Bact. *90* 1355–1364 1965
11. *Pooley H M & Shockman, G D* Relationship between the latent form and the active form of the autolytic enzyme of *Streptococcus faecalis*. J Bact *100* 617–624 1969.
12. *Scheie P O* Plasmolysis of *Escherichia coli* B/r with sucrose J Bact. *98* 335–340, 1969
13. *Shockman G D* The autolytic enzyme system of *St ptococcus faecalis*. II Partial characterization of the autolysin and its substrate Biochemistry *6* 1054–1063 1967
14. *Sjöström J E., Lindberg, M & Philipson L.* Competence for transfection in *Staphylococcus aureus*. J Bact *113* 576–585, 1973.
15. *Young, F E* Autolytic enzyme associated with cell walls of *Bacillus subtilis*. J. biol Chem. *241* 3462 3467 1966.

Acta path. microbiol. scand. Sect. B 83: 249–256, 1975

INFLUENCE OF OSMOTIC PRESSURE ON TRANSFORMABLE AND NON TRANSFORMABLE VARIANTS OF *NEISSERIA MENINGITIDIS*

3. Fragility of the Cell Membranes

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Jyssum, K. Influence of osmotic pressure on transformable and non transformable variants of *Neisseria meningitidis*. 3. Fragility of the cell membranes. Acta path. microbiol. scand. Sect. B, 83: 249–256, 1975.

Penicillin spheroplasts from competent (cp) and incompetent (cp^-) variants of *Neisseria meningitidis* Strain M1 were equally fragile as determined by the osmotic strength necessary for stabilization. Exponential phase cells adapted to growth under increased osmotic pressure and suspended in acetate or phosphate became osmotically fragile within 2–4 hours when stabilized by sucrose plus $MgCl_2$. Spheroplasts were formed during the succeeding 6–12 hours. Cells that were not adapted to growth at high tonicity but exposed to the stabilizing fluid under conditions leading to plasmolysis, developed osmotic fragility more rapidly but at the same time lysis of the membranes was activated. Under these conditions, membrane lysis occurred far more rapidly in the cp^- variant than in the cp variant. Membrane lysis was inhibited by $MgCl_2$ and enhanced by $CaCl_2$ in low concentration. The findings support the notion that osmotic stress in *N. meningitidis* activates membrane lysis as well as cell wall lysis, and that both activations are far more pronounced in the cp^- variant. But the relative protection of the cp variant cannot be due to higher tensile strength of the membranes.

Key words: *Neisseria meningitidis*; variants; osmotic pressure; cell membrane fragility

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Exposure of meningococci to conditions conducive to plasmolysis as well as to plasmoptysis leads to pronounced decrease in colony forming ability. The lethality is always significantly higher in the incompetent (cp^-) than in the competent (cp) variant (9). It has been demonstrated that exposure both to increased osmotic pressure and to decreased osmotic pressure activates cell wall autolysis in meningococci and that this activation is also far more pronounced in the cp^- variant (10). Since the autolytic enzymes of bacteria seem to represent integral parts of the wall structure (15, 17) this indicates that the cell wall complex of the cp^- variant is more easily damaged than that of the cp during osmotic stress.

Another consequence of osmotic stress seems to be a damage of the membranes. This assumption was derived from the observation that the cells loose their capacity to maintain an osmotic barrier (9) Also this membrane damage seems to be more pronounced in the *cp* variant under otherwise identical conditions.

The intention of the first part of this paper was to find out whether the relative resistance of the *cp*+ variant against damage during osmotic stress could be due to a higher tensile strength of the cytoplasmic membranes *per se* For this purpose, the lytic sensitivity of penicillin spheroplasts ("protoplasts") from the competence variants was compared at low osmotic strength. Subsequently the work is concerned with the influence of osmotic stress on the tensile strength as well as on the lysis of the cytoplasmic membranes. These experiments were mainly performed with spheroplasts or osmotically sensitive cells obtained after the action of native, autolytic enzymes (10)

MATERIALS AND METHODS

The methodology and experimental manipulations were the same as those described in the preceding papers (9 10) In similarity with these investigations, *N meningitidis* Strain M1 was generally used. In some experiments, Strain Ne15 of serogroup B, Strain Mad 1 of group A and strain P22 of group C (7) were also examined. Some procedures pertinent to the present work are listed below

Penicillin spheroplasts "Protoplasts" here generally termed spheroplasts, were prepared with penicillin in the presence of sucrose as stabilizing fluid. Portions of 12 ml Heart Infusion Broth (HIB Difco) were inoculated from an exponentially growing culture in the same medium and grown from A approximately 0.100 to A around 0.350 at 37 C on a reciprocal shaker The cells were centrifuged for 10 min at 7000 × *g* and resuspended *in HIB with 0.7 M sucrose and 0.05 M* $MgCl_2$ ("spheroplast broth") After resumption of growth (10) penicillin G 100 μg/ml was added. The culture was further incubated during shaking at 37 C and followed by measurements of the absorbancy and phase contrast microscopy After their release, the penicillin spheroplasts were spun down in the centrifuge Suspensions were prepared in 0.7 M sucrose with *0.015 M acetate and 0.05* M $MgCl_2$. In some experiments, acetate was substituted by 0.015 M phosphate buffer pH 7.8.

Determination of osmotic sensitivity Lysis curves were constructed on the basis of absorbancy measurements after exposure of spheroplasts to decreasing sucrose concentration (11) Samples of the spheroplast suspensions (0.2 ml) were diluted tenfold into 8 tubes with 1.8 ml portions of 0 05 M $MgCl_2$ plus 0.015 M phosphate buffer containing sucrose ranging in concentration from 0 to 0.7 M After incubation for 15 min at 37° C, all tubes were supplemented with 2 ml of 0.05 M $MgCl_2$ plus 0.015 M phosphate buffer containing sucrose in concentrations ranging from 1 4 to 0.7 M. The sucrose concentrations were chosen so that the final concentration in all tubes was 0.7 M. The suspensions were brought to room temperature and the absorbancy was read at 660 nm in the Bausch & Lomb Spectronic 20 photometer

Autoplasting The *N meningitidis* competence variants were grown in 12 ml batches of HIB as described for the preparation of penicillin spheroplasts. The cells were exposed to an osmotically protective solute system ("autoplasting system") as previously described (10) either directly or after previous adaptation to growth under increased osmotic pressure. 1) *Direct exposure* The exponential phase cells were collected in the centrifuge, washed once, usually with *0 15 M* acetate and suspended in the autoplasting system indicated in the actual experiment. 2) *Exposure after adaptation* The cells were collected in the centrifuge, resuspended in the "spheroplast broth" and for the production of penicillin spheroplasts and incubated until resumption of growth had occurred (10) Subsequently the cells were harvested in the centrifuge and resuspended in the "autoplasting system" under study

RESULTS

Osmotic fragility of penicillin spheroplasts. To determine quantitatively the membrane fragility of the *N meningitidis* competence variants, cells were grown in liquid media, adapted to growth under increased osmotic pressure, and then converted to spheroplasts by the penicillin method as described under Materials and Methods. The development of spheroplasts was followed by phase contrast microscopy Changes in the cellular morphology started to appear 2–3 hours after the addition of penicillin. The diplococcal unit became irregular and increased markedly in size. Globular units soon appeared. The diplococcal arrangement was al-

ways interrupted in completed spheroplasts. The number of globular enlarged cells steadily increased, and after 8–10 hours about 90 per cent of the population was thus changed. During prolonged incubation, the penicillin spheroplasts generally enlarged, some becoming very large. Old spheroplasts sometimes had a lunar-shaped vacuole at one side as described in cases of protoplasts of *Escherichia coli* (12)

Samples of spheroplast suspensions were subsequently exposed to decreasing concentrations of sucrose for 15 min subsequently the reduction in absorbancy was determined after readjustment to 0 7 M as described under Materials and Methods. In Fig 1 the lysis curves for the competence variants have been compared. No significant difference in osmotic sensitivity was observed.

As reported, it applies to most micro-organisms that magnesium ions were required for the stabilization of the penicillin spheroplast suspensions. Fig 2 shows that 0 02–0.03 M $MgCl_2$ is optimal for stabilization, and that there is no significant difference

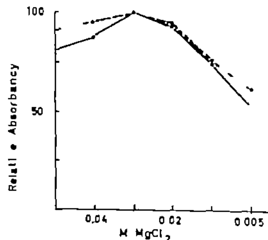
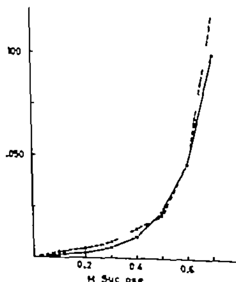


Fig 2 Stabilizing effect of $MgCl_2$ on penicillin spheroplasts from *N meningitidis* Comparison of competent (solid lines) and incompetent (dotted lines) variants. Suspensions of penicillin spheroplasts were prepared as described under Materials and Methods. Equal amounts were spun down in the centrifuge and resuspended in 0.015 M phosphate buffer with 0.7 M sucrose and $MgCl_2$ as shown. The data represent relative absorbancies at 660 nm after 10 hours.



g 1 Stabilizing effect of sucrose on penicillin heroplasts from N *meningitidis*. Comparison of mpetent (solid lines) and incompetent (dotted ies) ariants. Lysis curves were determined as scribed under Materials and Methods.

between the competence variants of Strain M1

In other experiments, competent and incompetent variants of the *N meningitidis* strains Ne 15 Mad 1 and P 22 (7) were similarly compared. In all the strains, penicillin spheroplasts of the competence variants had very nearly the same osmotic sensitivity

Autolytic development of spheroplasts It has previously been found that meningococci become osmotically fragile if suspended in appropriate stabilizing fluids (10) In Fig 3 lysis curves have been determined at various times during the development of osmotic fragility in suspensions of cells adapted to growth at increased osmotic pressure. Already after two hours, significant osmotic fragility could be demonstrated and, during the ensuing hours, the percentage osmotically sensitive cells steadily increased. The lysis curves became sigmoid-shaped and successively more like those obtained with penicillin spheroplasts, although the same steep increase in

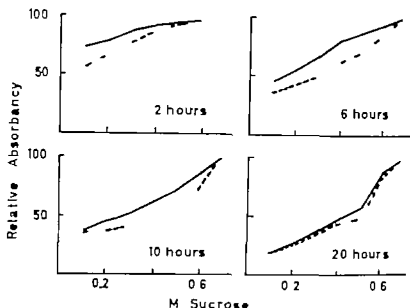


Fig 3 Autolytic development of osmotic fragility in *N meningitidis* suspensions after adaptation to growth in hypertonic media. Comparison of *cp* (solid lines) and *cp* (dotted lines) variants.—Exponential phase cultures were adapted to growth in HIB supplemented with 0.7 M sucrose and 0.05 M $MgCl_2$ (10) Suspensions were prepared in 0.015 M acetate with 0.7 M sucrose and 0.05 M $MgCl_2$ and incubated at 20° C. Lysis curves were determined at the times indicated as described under Materials and Methods.

stability between 0.5 and 0 7 M sucrose had not been attained even after 24 hours. As previously observed (10) it was found that the cp^- cells became osmotically sensitive more rapidly than the cp^+ cell under otherwise identical conditions. But the osmotically fragile part of the cell population was always *stabilized by the same concentration of sucrose* and there was no difference between the competence variants in this respect.

The concentration of Mg ions required for stabilization was the same as that necessary for penicillin spheroplasts, and both competence variants required the same concentration.

The suspensions were also examined by phase contrast microscopy during the development of osmotic fragility Very few spheroplasts were observed during the first 4–6 hours. Between 6 hours and 10 hours the number *increased to around 10 per cent of* the cells in the *cp* variant, and to between 30 and 40 per cent in the *cp* variant. During the ensuing hours, the number of spheroplasts steadily increased, but even after 20 hours several intact diplococci were observed, particularly in the *cp* variant.

Effects of osmotic shock on membrane stability In accordance with the previous findings (10) exposure of meningococci to increased osmotic pressure under conditions conductive to plasmolysis *increased* the rate of autolysis. This resulted in a more rapid development of osmotic fragility But it was observed that the fragile cells also lysed more rapidly in the stabilizing fluid upon prolonged incubation. This lysis was far more pronounced in the *cp* variant than in the *cp* variant. After 20–24 hours, no fragile cells were left in the *cp* suspensions (Fig. 4)

After exposure to osmotic shock, Mg ions still increased the stabilizing effect of sucrose substantially but by now higher concentrations seemed to be required and there was *a difference* between the *cp* and the *cp* cells, the latter requiring higher concentrations for stabilization (Fig. 5)

It has previously been found that Ca ions

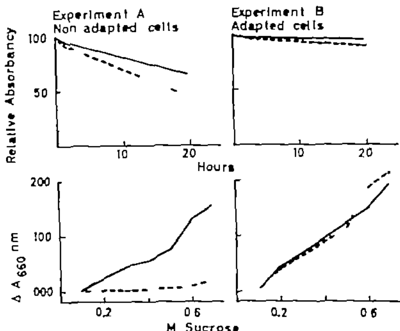


Fig 4 Effect of osmotic shock on the membrane stability in *N meningitidis*. Comparison of *cp* (solid lines) and *cp* (dotted lines) variants.—*Experiment A* HIB cultures in the exponential phase were harvested and exposed to 0.7 M sucrose with 0.05 M $MgCl_2$ and 0.015 M acetate. The decline in absorbancy was followed at 660 nm (top graph) during incubation at 30 C. After 20 hours, lysis curves were determined as described under Materials and Methods (bottom graph) *Experiment B* Exponential phase cultures were adapted to growth in HIB supplemented with 0.7 M sucrose and 0.05 M $MgCl_2$ (10) After resumption of growth, the cells were harvested and exposed to 0 7 M sucrose with 0 05 M $MgCl_2$ and 0.015 M acetate. The decline in absorbancy was followed (top graph) and lysis curves determined after 20 hours (bottom graph) as in Experiment A.

in low concentration counteract or abolish the stabilizing effect of acetate and sucrose (10) This effect was evident in suspensions of penicillin spheroplasts as well as in "autoplasts" Fig. 6 shows that the lysis of the latter when suspended in 0 6 M sucrose was enhanced by 0.005 M $CaCl_2$ even in the presence of 0.05 M $MgCl_2$. But the rate of lysis also depended on the buffer present in the stabilizing fluid. Phosphate buffer always resulted in more rapid lysis than acetate (Fig. 6) Tris buffer and acetate were interchangeable in this respect.

DISCUSSION

Ampicillin-induced spheroplasts have been prepared from *Neisseria flava* (6) but otherwise there are no descriptions of methods for the preparation of spheroplasts from Neisseria. In the present work, globular cells were obtained from *N meningitidis* by the penicillin technique. Although the globular cells obtained correspond well with the protoplasts described in other microbes, and are stabilized by approximately the same concentration of Mg ions (3 12) they may still possess cell wall material. They have thus been termed spheroplasts, restricting the term protoplast to cells in which all cell wall components apparently are absent (4) The diplococcal arrangement was lost when the bacteria were converted to spheroplasts. This probably indicates that the two partners after completed division are kept together by cell wall structures. In an early phase of the division cycle the two partners may have one membrane in common (2)

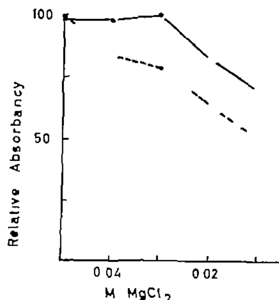


Fig 5 Effect of osmotic shock on the concentration of $MgCl_2$ required for stabilization in 0 7 M sucrose. Comparison of *cp* (solid lines) and *cp* (dotted lines) variants.—A HIB culture in the exponential phase was harvested in the centrifuge. Suspensions were prepared in 0.7 M sucrose with 0.015 M phosphate buffer and $MgCl_2$ as shown. The data represent relative absorbancies after 4 hours.

Although penicillin spheroplasts probably have preserved more or less cell wall material (3) it is likely that the lysis curves actually measure the osmotic stability of the cytoplasmic membranes (11) In this case, the present experiments demonstrate that there is no difference between the tensile strength of the intact membranes of the *cp* and the *cp* variants.

As in many other bacteria (8, 13, 14), osmotic fragility develops in *N meningitidis* in the presence of stabilizing fluid even without the addition of penicillin or lysozyme, due to autolysis of the cell wall (10) But in cells that have been adapted to growth in hypertonic media, the process is rather slow The pattern of lysis curves determined during the development indicates that the population is rather heterogeneous with regard to osmotic properties, and the fragile cells are only very slowly converted to globular spheroplasts. *Carson & Eagon* (5) reported that lysozyme-treated *Pseudomonas aeruginosa* retained their rod shape. Similar observations have been reported to apply to other gram negative bacteria (16) The term "osmoplast" has been used to designate intact, osmotically sensitive cells (1)

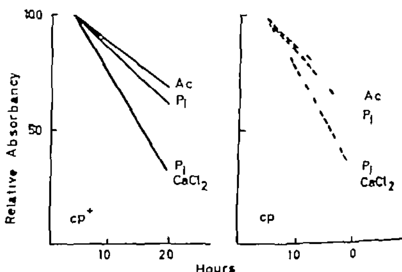


Fig 6 Lysis of *N meningitidis* in stabilizing fluid during prolonged incubation at 20 C. Enhancement of lysis by $CaCl_2$.—Exponential phase cells were harvested and suspended in 0 7 M sucrose with 0 03 M $MgCl_2$ supplemented with acetate (0 015 M) phosphate (0.015 M) and $CaCl_2$ (0 005 M) as shown. The data represent absorbancies at 660 nm relative to that observed after 4 hours incubation.

Autolysis as well as the development of osmotic fragility can be much enhanced by the exposure of the *N meningitidis* cells to osmotic stress (10) But the present experiments show that conditions conductive to plasmolysis also have rather drastic consequences for the stability of the membranes. The membranes, particularly those from the *cp* variant, now seem to require higher concentrations of Mg ions for stabilisation. This may be an indication that the membranes are indeed damaged (1) Still, even under optimal conditions, the membranes are lysed far more rapidly than the membranes in penicillin spheroplasts as well as in "osmoplasts" developed in cells adapted to growth under increased osmotic pressure. In accordance with the previous observations (10) it was observed that this lysis was enhanced by Ca ions in low concentration. It would thus seem that the membrane complex is disturbed during plasmolysis and that one consequence is the activation of a Ca^{++} dependent membrane lysis.

The present experiments substantiate the previous findings that the membranes of *N meningitidis* Strain M1 are damaged by plasmolysis and plasmoptysis (9) and that the *p* variant is relatively protected against this damage. Since the reason does not seem to be a higher tensile strength of the *cp* membrane *per se* other reasons must be considered (9)

Other experiments with the *N meningitidis* strains Ne15 Mad 1 and P22 (not published) along with the findings from penicillin spheroplasts reported above indicate that the existence of an association between membrane properties and competence in transformation may be a general phenomenon in this species.

The author is greatly indebted to Mrs *Lydia* Gahl for excellent technical assistance.

REFERENCES

1 *Asbell, M A & Eagon R G* The role of multivalent cations in the organization and structure of bacterial cell walls. Biochem. biophys. Res. Commun. 22 664–671 1966.
2. *Bideau, J.:* Étude de la structure de la couche superficielle de la paroi de *Neisseria meningitidis*. Thesis. Faculté mixte de médicine et de pharmacie de Marseille 1968.
3 *Birdsell, D C & Cota-Robles E. H.:* Production and ultrastructure of lysozyme and ethylenediaminetetraacetatelysozyme spheroplasts of *Escherichia coli*. J Bact. 93 427–437 1967
4 *Brenner S Dark, F A. Gerhardt P Jeynes M H., Kandler O., Kellenbenberger E., Klienberger-Nobel E. McQuillen, K., Rubio-Huertos M., Salton M R. J., Strange R. E., Tomcsik, J & Weilbull, C.:* Bacterial protoplasts. Nature (Lond.) 181 1713–1714 1958.
5 *Carson, K. J & Eagon R. C* Lysozyme sensitivity of the cell wall of *Pseudomonas aeruginosa*. Further evidence for the role of the non-peptidoglycan components in cell wall rigidity Can. J Microbiol. 12 105–108, 1966.
6. *Forkman A* The location of carbonic anhydrase in a strain of *Neisseria flava*. Acta path. microbiol. scand. Sect. B, 80: 460–466 1972.
7 *Frholm L. O Jyssum K & Bøvre K.* Electron microscopical and cultural features of *Neisseria meningitidis* competence variants. Acta path. microbiol. scand Sect. B, 81 525-537 1973.
8. *Joseph R. & Shockman G D.:* Autolytic formation of protoplasts (autoplasts) of *Streptococcus faecalis* 9790: Release of cell wall, autolysin, and formation of stable autoplasts. J Bact. 118 735–746, 1974
9 *Jyssum K.:* Influence of osmotic pressure on transformable and non transformable variants of *Neisseria meningitidis* 1 Survival and turbidity changes. Acta path. microbiol. scand. Sect. B, 82 484–494 1974
10. *Jyssum K.* Influence of osmotic pressure on transformable and non transformable variants of *Neisseria meningitidis* 2 Growth and induction of autolysis. Acta path. microbiol. scand. Sect. B, 83 240–248 1975
11 *Kent C., Krag, S S & Lennarz W J* Procedure for the isolation of mutants of *Bacillus subtilis* with defective cytoplasmic membranes. J Bact 113 874–883 1973.
12. *Lederberg J & St. Clair J* Protoplasts and L-type growth of *Escherichia coli*. J Bact. 75 143–160 1958.
13. *Mitchell P & Moyle J.:* Autolytic release and osmotic properties of "protoplasts" from *Staphylococcus aureus*. J gen. Microbiol. 16 184 194 1957
14 *Mohan R. R., Kronish, D P Pianotti R. S., Epstein R. L. & Schwartz B. S* Autolytic

mechanism for spheroplast formation in *Bacillus cereus* and *Escherichia coli*. J Bact. *90* 1355–1364 1965

15 *Shockman G D* The autolytic enzyme system of *Streptococcus faecalis*. II Partial characterization of the autolysin and its substrate. Biochemistry *6* 1054–1065 1967

16. *Lees J G*. Lysozyme lysis of Gram-negative bacteria without production of spheroplasts. J gen. Microbiol. *35* 313–317 1964.

17 *Young, F E*. Autolytic enzyme associated with cell walls of *Bacillus subtilis*. J. biol. Chem. *241:* 3462 3467 1966.

Acta path. microbiol. scand. Sect. B, 83: 257–274 1975

EPIDEMIOLOGICAL MARKERS FOR *PSEUDOMONAS AERUGINOSA*

5. Subdivision by Iterative Numerical Analysis of Isolates According to Lysotypes

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Bergan, T. Niemelä, T. & Gyllenberg, H. Epidemiological markers for *Pseudomonas aeruginosa*. 5. Subdivision by iterative numerical analysis of isolates according to lysotypes. Acta path. microbiol. scand. Sect. B, 83: 257–274. 1975.

A computer-based numerical approach to the allocation of *Pseudomonas aeruginosa* bacteriophage patterns has been presented. This rendered a useful identification of similar phage types. The grouping had epidemiological relevance. Grouping of phage typing patterns of *P. aeruginosa* by numerical analysis showed that the patterns of related isolates may differ in one strong lysotype reaction, occasionally even in more reactions. This parallels previous findings which have been based on studies of the reproducibility of the method and evaluations of differences in epidemiologically related strains from the same sources.

Key words: *Pseudomonas aeruginosa*; epidemiological markers; subdivision; lysotypes; numerical analysis.

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The detection of groups with similar phage typing patterns among large numbers of strains is often complicated because some variation in lysotype is permissible (3, 4); such data easily becomes impossible to easily survey.

It would seem reasonable that computer aided procedures could assist in grouping. The customary procedures of numerical taxonomy used to establish or test classifications are not entirely sufficient for the task of identification. Gyllenberg *et al.* (6, 7, 8) have recently developed an objective identification procedure. This strategy has previously been used to diagnose clinical bacterial isolates (7). Its performance has been examined by way of a simulation model (8).

Presently the procedure is used to detect groups of similar phage typing patterns among *Pseudomonas aeruginosa*.

MATERIAL AND METHODS

Bacteria and Phages

Included were 486 bacterial isolates of *P. aeruginosa* and 113 bacteriophages presented previously (1). Among the phages was a set of 24 phages included in a regular phage typing set which has been developed previously (2).

Typing Procedure

The phage typing procedure has been outlined elsewhere (2, 3).

The allocation strategy is based on two steps:

a) classification, and
b) identification.

The classification phase is constructive, consisting of the establishment of a reference system, referring ONU's (original numerical units = the elements to be grouped, here referring to a bacterial isolate) to distinct groups. Subsequently the ONU's are identified in terms of the classification systems, i.e. on the basis of their distances from the units (groups) of these.

To this avail, each ONU is charactermed by a series of phage sensitivity reactions. The reactions were scored according to usual conventions (4) and given individual numerical equivalents

Conventional score	Numerical score
−	0
±	1/3
+	2/3
+ +	1

On the basis of their total reaction patterns, groups of the most intimately related strains are formed in such a way that the difference between the character scores of an *ONU* and the mean reaction scores applying to the whole group the *group centroid* is as small as possible.

Establishment of a Classification (= Grouping) Reference System

The numerical classification scheme is reiterative and comprises the following stages

( ) First, a primary identification matrix is established. This entails the steps A–C

A. Initially the affinities between the ONU's of a suitable sample are expressed by squared Euclidean distances, d_{ij}

$$d_{ij} = \sum_{k=1}^{m} (x_{ik} - x_{jk})^2$$

where m is the number of characters, x_{ik} and x_{jk} the value of the unknown ONU's i and j for character k. The individuals are considered as points in an *m-dimensional* space.

B. On the basis of the resulti ... matrix, cluster analysis is perfor ... complete linkage cluster analysis (C ... pheno- gram at appropriate phe ... fines distinct clusters.

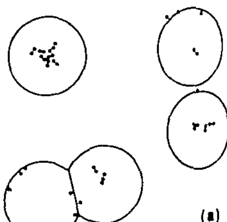
C. The ONU' of the N p ... constitute distinct clusters ... sional character space. The ... of each cluster are determined ... identification matrix showing ... between the h group centroid ... characters calculated. As an adj ... ference system is described by ... ance as a group heterogeneity

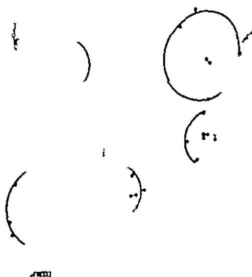
$$s_i = \frac{\sum d_h}{n} \quad i = 1 \ldots, h$$

where n is the number of individuals in group i and h pertains to all n_i individuals in group i. The distance d_{hi} between the centroid of group i and individual h is calculated, by

$$d_{hi} = \sum_{k=1}^{m} (x_{hk} - c_{ik})^2$$

(ii) The remaining ONU's are allocated on the bases of the identification matrix. Thereby each individual within certain limits (*vide supra*) becomes allocated to the group to which it has the shortest distance. During the stage of establishing a classification system, each group diameter is





equal (see Fig 1) After an initial allocation, some ONU's will be left outside the group limits. As the next step, it is investigated whether new groups may be established. A new group may be established when the distance of its group centroid to that of any other existing group exceeds given limit.

(iii) When a new group has been established, a new allocation cycle is required. For this purpose, previous as well as supplementary data on the whole material are utilized. During the new allocation cycle, some changes may occur in the constituency of both new and old groups. New group centroids are defined by the mean scores applying to all characters of the ONU's in the group.

(iv) The identification matrix has to be adjusted less as the number of reiterations increases, i the system approaches stability Few cycles are needed for completion, *i e* for the grouping of all elements, if the classification parameters define few groups distant from each other More cycles are required when groups are closer The computation by which to establish the classification system is interrupted only if the relative number of changes per reiteration cycle is reduced to certain level. Consequently the method involves system of continuous readjustment of classification and leads to an identification matrix containing the mean of each character of all ONU's in a group, *i e* values between 0 and 1 *Thence* follows the identification step.

Identification Strategy

The identification of the ONU's is also based on squared Euclidean distances between individuals and the group centroids in an *m*-dimensional space.

For identification, each group is described in terms of group centroid and intragroup variance, , required to determine group identity I each group, two radii are defined as multiples of the intragroup riance. These indicate whether an ONU is identified as a constituent of a group, a neighbour or whether it is further way from the group. The limits are fixed by the multiplication factors l and l (such that $l < l$) and the corresponding radii $l\,\sigma_i$ and $l_s\sigma$

Tw group spheres, I and J are overlapping if the distance between the group centroids is shorter than the sum of their identification radii, $d < l_1(+ _j)$

Accordingly four lev ls of identification are possible (Fig 2) an ONU may be

1 *Identified* when the distance between an ONU and group centroid is below $l_1\delta_i$ and the distance to the other centroids above $l_1\delta_j$

I termediate when an ONU belongs simultaneously to two groups by irtue of its absolute distance from both groups being below $l_i\sigma_i$ whereas the distance to other centroids is above $l_i\sigma_j$

3 *Neighbour* when the ONU is positioned between the limits $l_i\sigma_i$ and $l_s\sigma_i$

4 *Outlier* when the ONU lies outside $l_s\sigma_i$ of any group centroid.

ONU's occupying the Neighbour and Outlier positions have low and indecisive affinities to all groups in the identification matrix, *i.e.* are *sensu stricto* *nidentified* implying that the ONU in question cannot be allocated to any group on the basis of the existing *identification matri*

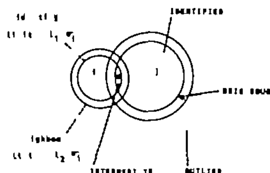


Fig 2 Illustration of identification level of the individual strains (indicated by solid dots) in relation to existing groups, I and J Group centroids have been indicated by "x An individual may be classified as Identified, Intermediate, Neighbour or Outlier according to affinity to group centroids as specified by the group variance σ_i or $_j$ and the limit multiplication factors l and l_s.

In order to facilitate comparison of different alternatives, the distances d_{ij} between the individual items and the group centroid have to be divided by the group variance. This gives a *normalized distan e* $D_{ij} = d_{ij}/\sigma_j$. Denoting the shortest distance D_a, and the next shortest D_b, the identification conditions can be described as follows.

Identified	I termediate	Neighbour	Outlier
(1) $D_a \leq l$ and (2) $D_b > l$	$D_b \leq l$	$l < D \leq l$	$D > l_s$

An *identification* matrix is a ([group centroid] × [character])-matrix consisting of the identification codes (characteristics) of each constituent of the reference system.

17*

The Relationship between Classification (Grouping) and Identification

A further clarification of the distinction between establishment of a classification system (identification reference system) and the identification is illustrated in Fig 1 If the individuals comprised in the series are classified in the grouping phase, the reference system obtained in this way can be used for the identification of the same individuals. It would be natural to support that each individual must be identified in the same group in which it is placed in the grouping stage. In fact, the situation is not always quite clear The basic differences between grouping and identification are presented below:

At the time of the *classification (grouping)* there is no definite information concerning the internal and mutual differences of the groups. For this reason the same maximum diameter is given to each group (Fig 1a) When the final group division is carried out, the internal variations of the groups can be examined.

In the *identification* situation, the internal variance of each group is known and these variances are used for the determination of the identification range. Accordingly the identification radius of a more homogenous group is smaller than that of a more heterogeneous group (Fig. 1b) At the stage of identification, the picture is much more complete because the differences in degree of suitability of the different group alternatives of each individual are brought out. In grouping, the situation is that the individual may either belong to or not belong to a certain group.

The adjustment of grouping is in a way an intermediate between grouping and identification. The internal dispersions of groups are known and at this stage the radii of the groups might be defined in relation to the variants previously observed. On the basis of concise empirical testing, however this hypothesis was rejected because it turned out that large groups had a tendency to expand, and small groups to shrink correspondingly The final result was thus a method by which the building and adjustment of the reference system gave groups the same maximum radii It was *only* at the identification stage that the observed group radii (= multiplication factor × variances) were taken into account.

Plan of Study

The classification of pseudomonas isolates according to phage sensitivity patterns was done in three slightly different approaches. They differed in the strains used for the establishment of a primary reference system and in the number of phage reactions used as variables.

1 Computation approach A The reactions of the 24 phages of the new phage typing set (2) were employed Since not all bacteria could be admitted to the classification procedure from the very beginning because of computer limitations, an initial reference system was established on the 162 consequtively oldest isolates from this hospital (Rikshospitalet) These were grouped by CLCA followed by the above described steps (I)–(n) *leading to the primary reference identification matrix.* Only then were the remaining 2/3 of the 486 strains admitted for reiterative semi-identification until classification stability was reached.

2 Computation approach B. This second alternative also included the 24 phages of the new phage typing set. A comparable number of bacteria as above was used to produce a primary identification matrix. In this trial, 162 strains were selected in such a way that every third strain was selected according to the strain numbers of the 486 strains. Afterwards, the remaining strains were admitted for identification.

3 Computation approach C As regards the third parallel, the reactions of all 113 phages were evaluated. Due to the limited capacity of the computer the bacterial allocation proceeded through three stages.

Initially the 121 bacterial isolates with the lowest strain numbers were grouped hierarchically by the complete linkage system of cluster analysis The initial reference system was based on 12 groups.

In the second stage, the individuals with strain numbers 1–306 were allocated. This resulted in a second stage reference system, which was subsequently employed with all 486 strains.

RESULTS

1 Computation Approach A

The first 162 pseudomonas strains rendered the CLCA phenogram of Fig. 3. From this, 16 clusters were selected for further processing as indicated by the V-marks on the stems of the phenogram clusters. The number 16 was chosen after studies of the internal variation of the clusters and the distances between the latter as indicated by preceding studies of groups formed at different phenon levels. This selection entails a subjective element. The aim is to achieve groups which are as homogeneous as possible while at the same time the distances between groups are long These two properties are in effect conflicting and accordingly a suitable compromise has to be made. The initial reference identification matrix grouped all but 91

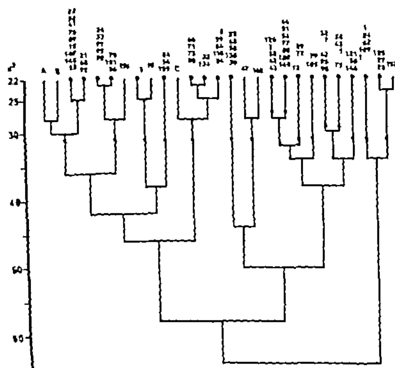


Fig. 3 Dendrogram of complete linkage cluster analysis of the initial 162 strains in Computation Approach A. The members of each cluster at the squared Euclidean distance level of 22 are shown in the Fig. by their identification numbers. Three large groups are indicated by letters A, B, and C and comprise the following strains.

A 2 4 8 11 12 13, 16, 18, 22 40, 50 60 61 64 80, 81 83 87 99 102, 103 112, 113 137 160

B 14 20 27 30, 31 33, 53, 56, 74 82, 83 86, 106 107 129 149 150, 151 155 161

C 10 13, 23 34 46, 52, 55 57 63 65 67 69 78, 90 94 108, 110 111 133 141 144;

The X-markers on 16 of the cluster stems indicate the phenon level of basic groups for the establishment of primary identification matrix.

TABLE 1 *Detection of Identification Reference Groups among Non-allocated Strains before the Final Identification of All Isolates*

Reference group	Group size	Computation A	Group size	Approach B
16			5	115 153 272 279 366
17	8	21 112, 212, 244 332, 362, 382, 383	5	17 24 62, 109 117
18	3	190, 281 351	4	437 441 472, 482
19	4	437 441 443, 472	3	140, 176, 177
20	3	115 122, 366		
Remaining ungrouped	73		77	

The Relationship between Classification (Grouping) and Identification

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RESULTS

1 Computation Approach A

The first 162 pseudomonas strains rendered the CLCA-phenogram of Fig 3. From this, 16 clusters were selected for further processing as indicated by the \-marks on the stems of the phenogram clusters. The number 16 was chosen after studies of the internal variation of the clusters and the distances between the latter as indicated by preceding studies of groups formed at different phenon levels. This selection entails a subjective element. The aim is to achieve groups which are as homogeneous as possible while at the same time the distances between groups are long. These two properties are in effect conflicting and accordingly a suitable compromise has to be made. The mutual reference identification matrix grouped all but 41

ctrix of Computation Approach A

					Bacteriophage							
12	13	14	15	16	17	18	19	20	21	22	23	24
.28	.00	.03	.00	.04	.03	.04	.03	.05	.07	.04	00	.01
.27	.00	.00	.02	.22	07	.04	.02	16	.05	.01	.08	.02
.07	11	11	1.0	14	00	.22	11	.22	.22	.00	.22	.00
.00	.00	.00	.00	.00	.25	.30	.50	.00	.25	.00	.08	1.0
10	.00	.00	.07	.05	.00	.01	.00	.04	.38	00	.07	.00
.80	.20	13	46	.20	.93	60	.00	1.0	80	13	.00	13
.00	.00	.00	1.0	.50	.83	.50	.00	00	1.0	.00	.00	.50
.61	.00	.00	.00	16	1 0	.05	16	.00	77	00	.00	00
75	.04	.04	.00	.00	.91	.04	00	.04	18	.00	.00	.00
.35	70	20	12	.04	12	.08	.00	.04	.20	.00	.20	.00
1.0	.33	.00	.00	16	1.0	1.0	00	.00	.00	.00	.00	.00
09	.00	.04	.01	19	.07	.00	.04	.04	.00	.00	.02	.01
.08	.00	.01	16	.35	75	.03	.00	.00	07	.00	.00	.00
11	.22	.00	.00	.00	.88	.33	.00	.00	00	.33	1.0	00
.04	14	.00	1.0	.28	.00	.81	.28	14	1.0	.00	.00	.00
.08	1.0	.50	1.0	75	.25	.58	75	75	.75	.00	.00	.00
11	.03	.00	.98	.29	.00	40	.00	.25	.30	.00	00	.00
.33	.00	.00	.33	1.0	46	.06	.00	1.0	00	.20	.00	.00
1.0	.00	.30	.00	.00	.25	.00	.00	1.0	.00	00	.00	00
.30	.01	.02	.00	91	.03	16	.00	.00	.06	03	02	00

are shown in Table 7 In Computation Approach A, two thirds of the strains were identified (*i.e.* as "Identified" or "Intermediate")

II Computation Approach B

The difference between this Approach and Computation A was only the selection of the 162 strains initially grouped by the hierarchical procedure. For obvious reasons, the initial identification matrices were not identical. In this trial, the strains were initially subdivided into 15 groups. Among 94 non-allocated strains, four new groups were detected (Table 1) One of these (*no. 18 in* Table 1) was identical to group 19 in Approach A, the others were dissimilar This reflects the fact that each Approach left different strains outside the reference system.

After the remaining 2/3 of the pseudomonas strains were admitted to the identification, 19 groups ultimately evolved. The final identification matrix differed from that of Approach A (not shown here) The mean intra-group distances are shown in Table 3 the matrix of centroid similarity in Table 8, and matrix of intermediate isolates in Table 9 Again, the group associations suggested by the matrix of intermediates is reflected by the lowest distance values in the centroid similarity matrix. Group homogeneity was comparable to that achieved by Computation Approach A.

III Comparison of Computation Approaches A and B

These two Computation Approaches did not render identical strain allocations between groups. At first glance, the results may appear to be dissimilar but upon a detailed analysis, the similarities dominate

As is seen from the distribution of the

TABLE 3 *Mean Squared Distances within Groups of the Final Identification Reference Systems*

Computation Approach	Reference groups 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
A	1.00	1 31	1 44	1.27	0 94	1 48	1 67	1 02	1.32	1.30	0.89	1.01	1.20	1.26	1 07	1 61	1.29	1 14	0.67	1 14
B	0 97	0.93	1 35	0.90	1.31	1 20	1 41	1.31	1.57	1.32	1.56	1 12	1.22	1 21	1.09	0 96	0.92	0 67	0.57	
C	5.84	9 78	5.54	9.51	4.07	9.87	11.09	7 48	11.86	8.16	9.58									

TABLE 4 *Matrix of Squared Distances between Group Centroids of Computation Approach A*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
2	1.06																		
3	2.23	1 49																	
4	1.96	2.21	2.62																
5	2.27	[illegible]	3.07	3.21															
6	6.94	5.90	6.97	7.84	7.22														
7	7 17	7.81	6.73	6.84	5.68	4.23													
8	5.61	4 10	5.61	5.48	5 16	2.80	4.89												
9	2.42	2.91	4.59	3.92	4.87	3.61	5.05	2.13											
10	3.86	2.55	3.61	4.53	4.81	4.34	7 53	2.30	2.38										
11	5.63	5.85	6.89	5 90	5 47	4 10	6 05	3.55	3 14	3.65									
12	1 13	1 65	2 73	2 16	2 66	6 49	6.01	4 47	2.28	2.86	4.52								
13	2.24	2.96	4 52	3.90	3.90	4 15	4.22	3 12	1 16	3.60	3 79	1 46							
14	3.31	3.54	4.68	4.28	5.69	5.36	6.25	3.90	1 74	3.62	4.81	3 13	2.02						
15	5 42	5.92	3 76	5.51	2.37	7.60	4 22	7 10	7.88	7.37	7.22	5 79	6.80	8.63					
16	7 74	7.65	5.85	7.37	5 03	5.98	5.08	7.50	8.43	6.47	6.52	6.27	6.74	9.27	3.29				
17	1 40	2.25	1.56	3.33	3.33	6.21	5 44	6.96	4 07	5.22	6.72	2.31	2.88	4 67	3 66	5.58			
18	2.64	3 15	3.90	4.99	5.58	5 23	6.99	6.78	5.56	5.52	6.46	2.87	2.75	4.85	7.22	6.50	2.11		
19	6 93	5.53	6.98	8 01	8.67	4 71	8.50	5.19	4.35	4.05	6.25	5 91	6.63	6.74	11.68	10.12	8.54	6.86	
20	0 87	1.58	2.95	2.91	2.71	7.27	7.24	6.09	3.50	4 62	3 60	1 72	2 47	4.38	5.40	6.05	1 76	1.91	8.26

distances, Fig. 4 the variation in inter-strain affinity was found to be approximately the same in both groups of the 162 initial strains. More similarity might have been expected among the strains of Approach A, since they all derived from the same hospital. This would have rendered a more squewed distribution with the mode value more to the left of the mean. By way of comparison, it is seen in the Fig 5 that the non-classified 91 respectively 94 strains had fairly symmetrical distribution curves representing squared distances, indicating a lesser similarity within these strains.

A detailed scrutiny of strain classification has to be carried out to find how the two Computation Approaches A and B compare. Table 10 shows that group 5 in computation

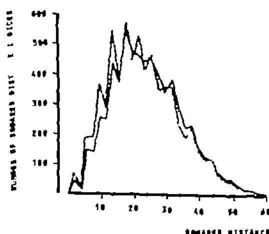


Fig 5 Distribution of squared distance indices between all 91 primarily unidentified strains in Computation Approach A (solid line) and 94 primarily unidentified strains in Computation Approach B (broken line)

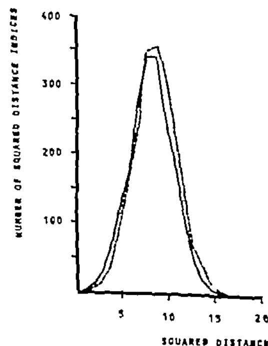


Fig 4 Distribution of squared distance indices between all 162 strains for Computation Approach A (solid line) and Computation Approach B (broken line)

Approach A, A.5* is identical to B·4 and mostly comprises strains isolated from this hospital (25 out of the 26 group constituents)

Similar strong group associations between the two Computation Approaches are seen to apply to the constituents of A.8, A 14 A 15 A 17 A 19 and A:20 The one strain of A.8 that was not identified as B·6 was a neighbour thereto The strong affinity between A 17 and B 10 is also reflected in the 5 B 10 strains in A·0 which were neighbours to A 17 The 5 A.20 strains not identified together with the majority of the A.20 constituents in B 12 were neighbours to the latter group.

It was interesting to note that approximately the same number remained unidentified (Neighbours and Outliers) in both Approaches.

In cases where A-groups were divided between more B-groups, only a few B-groups were involved. For instance, almost 2/3 of the A 1 strains were of B 1 and nearly 1/3 in

* In the following, a capital letter and numeral will be used to designate Computation Approach and respective group e.g. A:5.

TABLE 3 *Mean Squared Distances within Groups of the Final Identification Reference Systems*

Computation Approach	Reference groups																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
A	1.00	1.31	1.44	1.27	0.94	1.48	1.67	1.02	1.32	1.30	0.89	1.01	1.20	1.26	1.07	1.61	1.29	1.14	0.67	1.14
B	0.97	0.93	1.35	0.90	1.31	1.20	1.41	1.31	1.57	1.32	1.36	1.12	1.22	1.21	1.09	0.96	0.92	0.67	0.57	
C	5.84	9.78	5.34	9.51	4.07	9.87	11.09	7.48	11.86	8.16	9.58									

TABLE 4 *Matrix of Squared Distances between Group Centroids of Computation Approach A*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
2	1.06																		
3	2.23	1.49																	
4	1.98	2.21	2.62																
5	.27	2.84	3.07	3.21															
6	6.94	3.90	8.97	7.84	7.22														
7	7.17	7.81	6.73	6.84	5.68	4.23													
8	5.61	4.10	5.61	5.48	5.16	2.80	4.89												
9	2.42	2.91	4.59	3.92	4.87	3.61	5.05	2.13											
10	3.86	2.55	3.61	4.53	4.81	4.34	7.33	2.30	2.38										
11	3.63	5.83	6.89	3.00	5.47	4.10	6.05	3.55	3.14	3.65									
12	1.13	1.63	2.73	2.16	2.66	6.49	6.01	4.47	2.28	2.86	4.52								
13	2.24	2.96	4.52	3.90	3.90	4.15	4.22	3.12	1.16	3.60	3.79	1.46							
14	3.31	3.54	4.68	4.28	5.69	5.36	6.25	3.90	1.74	3.62	4.81	3.13	2.09						
15	3.42	3.82	3.76	5.51	2.37	7.60	4.22	7.10	7.88	7.37	7.22	5.79	6.80	8.63					
16	7.74	7.65	5.65	7.32	5.03	5.98	5.08	7.50	8.43	6.47	8.52	6.27	6.74	9.27	3.29				
17	1.40	2.25	1.56	3.33	3.33	6.21	3.44	6.98	4.07	3.22	6.72	2.31	2.88	4.67	3.66	5.58			
18	2.66	3.15	3.90	4.90	3.56	3.23	6.99	6.78	3.56	3.52	6.46	2.87	2.75	4.83	7.22	4.50	.11		
19	6.93	5.33	6.98	8.01	8.67	4.71	8.50	3.19	4.35	4.05	6.35	5.91	6.69	6.74	11.68	10.12	6.54	6.88	
20	0.87	1.58	2.95	2.91	2.71	7.27	7.24	4.09	3.50	4.62	3.60	1.72	2.47	4.38	5.40	6.05	1.76	1.91	8.26

…cording to Lysotyping with … Bacteriophage Typing Set Comprised of 24 Bacteriophages. Evaluation …pproach A

Strains												
28	31	36	40	56	59	60	61	64	74	76	82	83
230	234	235	236	251	254	256	259	260	267	268	271	277
380	381	387	392	398	399	401	405	406	411	415	418	422
445	447	448	449	451	456	460	467	468				
156	158	164	188	193	194	197	242	243	250	269	310	311
66	67	69	73	78	90	108	141	144	203	205	207	214
307	309	404	428	466	471	478						
211	213	215	231	238	247	283	320	385	407	409	410	413
249	266	299	336	416	461	470	474	475	479	483	484	
244	273	314	333	350	355	356	357	362	372	397		
161	165	171	184	195	224	240	241	258	261	262	264	265
368	373	384	389	391								

When studied separately one additional group of three members could be created among these. When all 486 strains were identified in terms of the secondary 13-group identification matrix, 44 individuals remained unidentified. It turned out, however that 3 of the groups were overlapping to such an extent that they had to be combined. Consequently the number of groups was reduced to 11 The final identity limit was 1 4 σ_i and the limit between Neighbour and Outlier 1.8 σ Subsequently 74 ONU's remained unidentified.

The matrix of squared distances between group centroids of Computation Approach C appears in Table 12. Whereas the lowest values in the two previous Approaches were less than one the lowest figure was 4 15 in this case. The higher values are mainly due to the larger number of characters employed presently (through addition of more figures) Accordingly also mean squared distances within groups were larger if this Computation Approach was used (Table 3) The lowest values in the centroid similarity matrix are reflected in a relatively high number of intermediate strains (Table 13) Sharing 71 intermediate strains, groups 1 and 10 were particularly overlapping These were all the elements of group 10 and 70 per cent of the members of group 1 Analogously 12 of the 13 constituents of group 6 were intermediate to group 7

The identification matrix based on 113 phages has been interpreted in terms of the new phage typing set as listed in Table 14

TABLE 5 *Identification According to Reference Group for 486 Isolates of Pseudomonas by Comp...*

Identification group	Number of strains	Strains									
1	88	2	4	8	11	12	13	14	16	18	[illegible]
		87	102	107	185	187	189	190	196	218	[illegible]
		297	316	340	343	348	358	370	371	374	[illegible]
		423	424	425	426	433	434	435	436	438	[illegible]
2	29	3	7	26	35	70	91	92	93	101	[illegible]
		321	327	332	335	342	386				
3	7	100	116	173	228	293	306	313			
4	10	5	191	305	360	403	420	421	467	444	[illegible]
5	26	10	13	23	34	46	52	55	57	63	[illegible]
		216	253	481							
6	4	48	138	139	287						
7	2	47	162								
8	5	6	126	132	142	143					
9	17	37	44	51	77	88	96	120	178	208	[illegible]
10	8	29	72	97	123	239	337	339	369		
11	2	79	105								
12	31	27	30	32	53	129	163	174	181	186	[illegible]
		414	431	440	430	452	459	476	486		
13	22	1	49	75	168	179	201	221	223	245	[illegible]
14	2	121	146								
15	4	17	62	109	117						
16	3	125	127	128							
17	21	80	81	85	99	112	113	137	166	192	[illegible]
18	4	220	281	282	351						
19	3	437	472	482							
20	38	41	105	106	118	149	150	151	153	155	[illegible]
		270	278	291	292	294	296	301	341	346	[illegible]

* Unidentified strains, Neighbours and Outliers have not been listed in the Table.

B 16. Two thirds of the A 19 and B 2 strains were either shared or were neighbour to each other. The three A 16 strains were either classified as B 13 or were Neighbour or Outlier thereto.

In routine phage typing, scores above a certain level 0.666 corresponding to a + or a + + reaction, are included in the sensitivity pattern. In Table 11, the identification matrix has been interpreted as traditional phage sensitivity. The associations between reference groups in Computation A and Computation B have been indicated by solid and interrupted arrows according to major or minor overlapping.

II. Computation Approach C

Above, the results obtained with Twenty four phages have been described. How a character space of 113 phages would subdivide the bacterial strains was also investigated. In this case, only the first 1/4 of the bacteria were included in the hierarchical CLCA grouping. Twelve groups emerged at a level where the intra-group squared distances were below 30.00. The remainder was grouped by two stages.

Application of the primary reference system of the first 306 bacteria resulted in a secondary reference system which was ultimately applied to all 486 strains.

After two iterations in the series of the 306 individuals, only 27 remained ungrouped

TABLE 7 *Results of Identifying 486 Strains of* Pseudomonas aeruginosa *According to Bacteriophage Sensitivity Reached in Three Alternative Computation Approaches*

Computation Approach	Identification level	Identification matrix group 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Total
A	Identified	33	15	6	9	26	4	2	5	8	8	2	18	12	2	4	3	14	2	3	26	202
	Intermediate	53	14	1	1					9			13	10				7	3		12	124
	Neighbour	20	13	7	4	4	5	6	1	10	4		10	7	2	3	3	12	2	1	11	125
	Outlier		4	2	2		6	3		5	1			1		1	3	3	1		1	35
	Subtotal Ident. + Interm.	88	29	7	10	26	4	2	5	17	8	2	31	22	2	4	3	21	4	3	38	328
	Total	108	46	16	16	30	15	11	6	32	13	2	41	30	4	8	9	38	7	4	50	486
B	Identified	14	5	3	26	26	6	3	2	17	22	7	21	2	4	8	11	4	3	3		189
	Intermediate	59	11	3		4				6	7		17			6	16					129
	Neighbour	14	3	11	5	13	2	4		33	12	3	20	1	3	4	8	2	1			139
	Outlier			1		2		3		12	2	1		3	3	1		1				29
	Subtotal Ident. + Interm.	73	16	8	26	30	6	3	2	23	29	7	38	2	4	14	27	4	3	3		318
	Total	87	19	20	31	45	8	10	2	66	43	11	58	6	10	19	35	7	4	3		486
C	Identified	1	20	30	2	3	5	48	5	24	33	18										189
	Intermediate	104	4	2	1		7	27			33	25										221
	Neighbour	1	11	2				8		5	5	7										41
	Outlier		4	2			1	9		11		8										35
	Subtotal Ident. + Interm.	105	44	32	3	3	12	75	5	24	66	43										410
	Total	106	59	38	3	3	13	92	5	40	71	58										486

TABLE 8. *Matrix of Squared Distances between Group Centroids of Computation Approach B*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
2	1.10																	
3	1.91	2.87																
4	2.14	3.40	4.12															
5	2.07	1.54	3.98	4.15														
6	5.24	4.21	6.54	5.21	2.74													
7	7.74	6.74	6.62	7.37	5.01	2.88												
8	5.20	4.44	6.46	6.17	2.87	4.10	4.34											
9	2.12	2.67	3.43	3.80	2.29	2.63	4.88	3.74										
10	1.18	2.08	3.20	2.97	2.98	3.94	6.80	6.14	2.98									
11	4.39	4.22	6.20	5.26	2.48	3.27	3.58	3.70	4.19	3.02								
12	1.04	2.09	2.54	2.73	2.51	3.66	8.52	5.63	2.83	1.76	4.40							
13	6.60	7.95	7.65	4.98	6.04	6.39	3.74	3.75	6.34	5.09	3.86	6.33						
14	3.56	4.13	5.19	3.96	5.54	5.70	4.49	6.26	4.89	4.03	5.39	4.40	6.12					
15	1.57	3.03	2.51	3.01	3.92	4.96	6.95	6.20	1.71	2.29	5.38	1.74	6.57	3.48				
16	1.01	1.54	2.85	3.20	2.64	5.29	7.89	5.61	1.96	1.76	5.41	1.70	7.94	4.54	2.71			
17	5.87	7.10	7.03	2.64	7.75	7.63	7.19	8.17	6.94	4.22	4.71	3.75	3.94	5.49	6.01	6.91		
18	7.00	6.34	8.21	8.69	5.73	4.25	6.16	6.77	3.75	7.99	8.66	7.94	9.58	7.91	6.11	6.48	12.15	
19	4.90	4.02	3.90	5.44	4.22	4.37	8.17	5.92	4.07	5.59	6.71	5.65	8.06	6.31	4.37	4.22	8.96	5.45

TABLE 9 *Matrix of the Number of Intermediate* Pseudomonas aeruginosa *Isolates between the Various Groups of the Final Identification Reference System of Computation Approach B*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
2	6	×																
3	4		×															
4	1			×														
5	8	4			×													
6						×												
7							×											
8								×										
9	6								×									
10	36									×								
11											×							
12	11				1					1		×						
13													×					
14														×				
15	14											5			×			
16	13	4								1		5				×		
17																	×	
18																		×
19																		

tions. For instance, the phage type B 12 (group A:9) and related patterns (A 11 A 13 B 16, and B 12) were the cause of nosocomial outbreaks in the departments of neurology and paediatrics, and in the surgical department A (4) The phage group of A 13 or B 17 was found in a series of tracheotomized patients treated with respirators in the surgical department A (4) Thus, the procedure for group construction and identification has rendered an epidemiologically relevant subdivision of the *P aeruginosa* strains.

The results of Approaches A and B may also contribute to the understanding of associations between similar phage types, as shown in Table 11 The previous conclusion based on reproducibility studies (3) and on serial isolates from the same patients (4) namely that nosocomially inter-related strains could vary in one strong reaction and, with *P aeruginosa* occasionally even in more reactions, is paralleled by the balance of associated patterns shown in Table 11 The strains have been referred to groups in the two Approaches which usually differed at the most in only one strong reaction. By reference to weak reactions, ± scores, additional information significant for the evaluation of phage pattern resemblances can be obtained. It is important to note that Computation Approach B rendered four phage patterns, B.2 B:8 B 11 and B 14 which had no apparent correlate in Approach A. Similarly it appeared that A 7 was not represented in the Computation B identification matrix.

In conclusion, this Approach to numerical treatment of phage typing data on *P aeruginosa* succeeded in detecting groups of nosocomially related strains. In order to obtain the most relevant identification reference system, it should be based on all strains under scrutiny or on unbiased selections from such material. The presently employed limits for identification and outlier status appears suitable for the separation of pseudomonas phage types.

REFERENCES

1 *Bergan T.*, Comparison of numerical procedures for grouping pseudomonas bacterio-

TABLE 10. *Group Correlation of Computation Approaches A and B*

Computation Approach A	Computation Approach B 0‡	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	Total
0	122*		5			1		3	1	5	5	7		1	4		5			5	160
1	1	57	5	3													22				88
2	5	5								7						12					29
3	2										5					2					7
4	4	1		5																	10
5					26																26
6							2														4
7	2																				2
8	1						4														5
9	6		6			5															17
10	5									5											8
11	1								1												2
12	7	10				2				6	1		5								31
13						22															22
14	2																				2
15																		4			4
16	2													1							3
17	1										20										21
18	2										2										4
19																			3		5
20	5												33								38
Tot.	168	73	16	8	26	30	6	5	2	23	29	7	38	2	4	14	27	4	3	5	488

‡ Group 0 contains "Neighbours" and "Outliers" Number of *Pseudomonas aeruginosa* strains.

TABLE 11 *Associated Reference Groups of the Final Identification Matrix of Computation Approaches A and B as Indicated by Mean M for Reactions**

Computation A		Computation B	
Identification group	Sensitivity pattern	Sensitivity pattern	Identification group
(1)	NT**	NT	(1)
(2)	4/A1	4/11 15	(15)
(3)	4/A1 A5		
(4)	19/14	14 19/4	(3)
(5)	7/2 16	7/2 16	(4)
(6)	4 5 8 12 15 16/13	4 8 12 13 15 16 18/7 10	(7)
(7)	5 7 12 16/2 6 11 13 19		
(8)	4 12 16/2 8	4,8 12 16/5	(6)
(9)	8 12	8	(16)
(10)	4 8 9	4 8	(9)
(11)	8 12 13/3,5 9		
(12)	A2/A1		--
(13)	12/11	12	(5)
(14)	12 18/4 13 17		
(15)	2 7 13 16	2,7 13 16/11 14	(17)
(16)	7 9 11 14 15,16/5 10 13	7 9 13,15/6 11 12 14 16	(13)
(17)	A5/13	A5	(10)
(18)	11 15/8 12	1 7 8 12 15	(19)
(19)	3 4 5 6 8 15/10	3 4 5 6 8 15/10	(18)
(20)	11	11	(12)
		A4	(2)
		12 13 15/11	(11)
		13 15 16 18/4	(14)
		9 12 13/3,8 17	(8)

Associated group patterns are indicated by arrows, dotted lines corresponding to weak affinities solid lines are used when more than 80 per cent of the strains within groups were identical. The identification group designations have been indicated in parentheses to avoid confusion with the reaction pattern codes. Only the reactions to the 19 phages of the basic phage typing system are shown, unless no strong reaction to any of these appeared. In the latter instance, the auxiliary set is utilized (phages A1 A5 *vide* reference (2))

phages according to lytic spectra. Acta path. microbiol. scand. Sect. B, 80 55–70 1972.

2. *Bergan T* A new bacteriophage typing set for *Pseudomonas aeruginosa*. 1. Selection procedure. Acta path. microbiol. scand. Sect. B, 80 177 188, 1972.

3. *Bergan, T & Lystad A.:* Reproducibility in bacteriophage sensitivity pattern of *Pseudomonas aeruginosa*. Acta path. microbiol. scand. Sect. B, 80 345–350, 1972.

4. *Bergan T.*. Epidemiological markers for *Pseudomonas aeruginosa*. 3 Comparison of bacteriophage typing, serogrouping, and pyocine typing on a heterogeneous clinical material. Acta path. microbiol. scand. Sect. B, 81: 91–101 1973

5. *Blair J E. & Williams R E. O.,* Phage typing of staphylococci. Bull. Wld Hlth Org. 24: 771–784 1961

6. *Gyllenberg, H G Niemelä, T K & Niemi*

TABLE 12. *Matrix of Squared Distances between Group Centroids of Computation Approach C*

	1	2	3	4	5	6	7	8	9	10
2	6.00									
3	20.44	13.32								
4	35 47	24.24	27 73							
5	37 13	26.38	19 73	27.38						
6	18.37	13.95	24 43	16.76	31.54					
7	8.32	9 29	25.51	19 22	31 60	7.03				
8	23.61	17 16	21 76	17.30	23.61	10.39	13 42			
9	29.86	17.39	24.95	9.56	20 49	12.74	13.81	13.06		
10	4 15	8.04	18.89	28.42	35.67	14 17	11.05	21.33	28 03	
11	6.64	5.67	15.53	22.93	29 18	19.88	10.08	22.25	18.79	9.9

TABLE 13 *Matrix of the Number of Intermediate* Pseudomonas aeruginosa *Isolates between the Various Groups of the Final Identification Reference System of Computation Approach C*

	1	2	3	4	5	6	7	8	9	10
2	20	×								
3		2	×							
4				×						
5					×					
6						×				
7	18	12				12	×			
8								×		
9				1		3	2		×	
10	71	14					3			x
11	28	22					6			1

TABLE 14 *Final Identification Matrix of Computation Approach C Interpreted in Terms of Typing Results with the New Phage Typing Set Used in Computation Approaches A and B*

Group	Phage pattern
1	NT*
2	/8
3	7/2, 16
4	4 8, 12, 13 16/7 9 10 15 18
5	7 9 10 11 14 15 16/3, 13
6	12/7 8
7	12/8
8	12/11 16
9	4 8 12/9 11 13
10	/8, 11
11	A1/4

*NT = non-typable.

J S A model of a tomatic identification of Streptomyces Postepy Higieny i Medycyny Doswiadczalnej (Poland) In press.

7 *Gyllenberg, H G Niemelä T K., Jousimies, H., Hatakka A Sederholm H & Aumi, J. S* Development of reference systems for automatic identification of clinical isolates of bacteria. Archives of Immunology and Experimental Therapy (Poland) In press

8. *Niemelä, T K & Gyllenberg, H G.:* Simulation of a computer-aided continuously correcting classification method. In Bacterial Identification with Computers (Academic Press, London) I press.

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RUBELLA IN ICELAND

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Tómasson, H. Ó. & Ogmundsdóttir H. M. Rubella in Iceland. Acta path. microbiol. scand. Sect. B, 83 275–284 1975.

Rubella has been a notifiable disease in Iceland since 1888. In this century rubella epidemics of increasing size have occurred at intervals of 5–10 years. The disease has spread throughout the country. About 75 per cent of the patients were below 15 years of age at the time of the last two epidemics. Females account for 51–61 per cent of the reported cases. In this first study of the sero-epidemiology of rubella in Iceland, epidemiological data and blood samples were collected from 1464 women from 15 locations. The group of 1484 women was further divided into 12 age-groups (range 5–49 years) so as to make correlations with the last six epidemics. The immunity status was determined by measuring HI-antibodies in serum. 67.8 per cent of the sera were positive (HI-titre >20). 81.1 per cent of women of child-bearing age (16–42 yr.) were seropositive. Two of the age-groups have gone through only one epidemic '63–'64. Children who were then 1–5 yr. old have an immunity ratio of 46.1 per cent, this ratio being 73.5 per cent in the case of children who had attained school age. The immunity ratio is slightly lower among subjects from rural districts than among those from urban districts. Most of the women have caught the disease at the time of the first or second epidemic to occur in their lifetime. 11.6 per cent of persons giving a positive history were seronegative, 52.1 per cent of seropositive subjects gave a positive history. According to population statistics and the serological results it is estimated that appr. 1 out of 7 cases was reported in the epidemic '63–'64.

Key words: Rubella; Iceland.

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The epidemiology and teratology of rubella in Iceland have been considered previously by several Icelandic authors (1, 11, 12, 13, 14, 15). Their work has been based purely on clinical grounds. The study presented here is the first serological survey on rubella antibodies in Iceland. Similar surveys have been undertaken in many countries. Official health statistics have everywhere been found to give very unreliable information about the epidemiology of rubella (2). The results of this survey provide the necessary background for the consideration of preventive measures against rubella such as vaccination.

Epidemiology. In 1888, rubella became a notifiable disease in Iceland. The earliest record on rubella was 1883–1884 (14, 16). It is not certain that epidemics in 1887–1889 and 1895–1897 actually were rubella, but it can be taken for granted that a rubella epidemic occurred in 1906–1907 (5, 10, 14, 16, 17). Since then, rubella epidemics have been recorded at intervals of 5–10 years, see Fig. 1 (5, 6, 7).

Commencing 1925–1926, the epidemics

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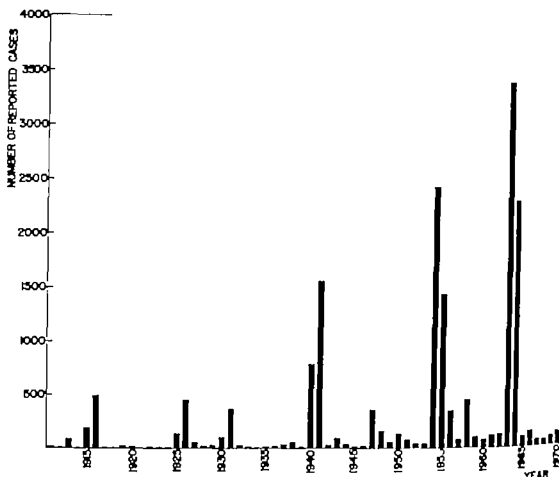


Fig 1 Reported cases of rubella in Iceland 1911–1970.

generally exhibit a similar pattern, a slow start in the spring or summer increasing in the fall, reaching a peak in midwinter and ebbing out towards the spring

It is not detectable that the epidemics have followed a certain route around the country (as in Taiwan (3)) The epidemics '25–'26 and '30–'31 both commence in the north, but since then they all begin in Reykjavík, the capital with now nearly half of the total population. The spread around the country often seems to be coincidental rather than continuous and to be determined by communication and activity in the districts at each time. In each epidemic some locations escape, but on the whole the disease does not seem to have bypassed any larger area. Table 1 gives an idea about the distribution of each epidemic since '25–'26 in different parts of the country and shows which epidemics reached the districts selected for this study

From Fig 2 it can be seen that the epidemics since '25–'26 have increased in size in terms of per cent of the whole population, with the exception of '30–'31 and '47–'48.

The distribution according to age is shown in Table 2. In the last two epidemics, the proportion of young people is larger than before.

In all epidemics there is a slight female preponderance, females are 51.3–51.4 per cent of patients below 15 yr. of age, 51.0–59.7 per cent in the age-group 15–19 yr. and 51.0–60.9 per cent among patients 20 yr. and older (not until 1954 were patients younger than 15 yr. classified by sex) This difference between the sexes is significant only in the

TABLE 1 *Spread of Rubella Epidemics in Iceland*

Iceland	Epidemics '25–'26	'30–'31	'40–'41	'47–'48	'54–'55	'63–'64
South-West	2/3†	2/3	3/4	2/4	3/4	5/5
Reykjavík	+	+	+	+	+	+
West	0/6	2/6	4/6	1/6	2/6	3/6
Kleppjárnsreykjah.	–	–	+	–	–	–
Stykkishólmsh.*	–	+	+	–	+	+
'estfjords	2/12	0/12	8/12	1/12	5/11	8/12
Reykhólahérad*	–	–	–	–	–	–
Ísafjördur*	+	?	+	+	+	+
rth West incl kureyri	4/8	0/8	7/9	0/9	8/10	8/10
Akureyri*	+	–	+	–	+	+
'orth-East	3/4	2/4	3/4	0/4	3/4	4/5
Thingeyjarsýslur*	+	+	+	–	+	+
East	2/9	3/9	3/9	4/9	6/10	7/10
Vopnafjördur	+	–	?	+	?	–
Seydisfjördur*	+	+	–	+	+	+
Hafnarhérad*	–	–	–	–	+	–
South	0/6	1/6	4/6	2/7	5/8	8/9
Kirkjubæjarhérad	–	+	?	–	–	–
Víkurhérad*	–	–	–	+	+	+
Selfoss*	–	–	–	–	+	+
No. of districts recording a rubella epidemic for the first time after 20 years	10	5	17	2	6	10

† no. of districts recording rubella/total no. of districts in this area.
district chosen for study in the area. Occurrence of rubella epidemic in district is shown by + Information is often unreliable. ? no information.

TABLE 2. *Age Distribution in Rubella Epidemic in Iceland*

Epidemic	Age in years <15	15–19	≥20	Total
'25–'26	359 (62.2 %)	218 (37.8 %)		577
'30–'31	242 (51.5 %)	77 (16.4 %)	151 (32.1 %)	470
'40–'41	1379 (58.8 %)	471 (20.1 %)	497 (21.2 %)	2347
'47–'48	335 (65.2 %)	75 (14.6 %)	104 (20.2 %)	514
'54–'55	2947 (75.7 %)	452 (11.6 %)	496 (12.7 %)	3895
'63–'64	4477 (73.6 %)	735 (12.1 %)	870 (14.3 %)	6080

e χ^2 for patients younger than 15 and 15 years and older is 339.5, i.e. $p<0.1$ per cent. Note Before 62, age-groups were specified in Public Health records by: 0–1 1–5 5–10 etc., but since 1962 by 0–1 4 5–9 etc.

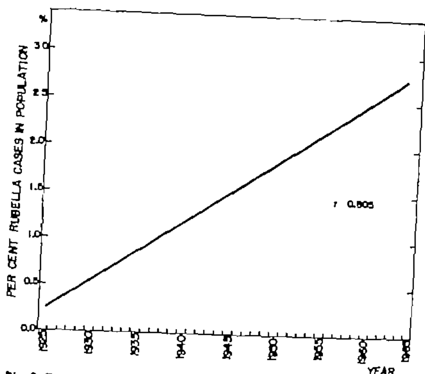


Fig. 2 Proportional size of rubella epidemics in Iceland since 1925–1926, and line of regression, b: 0.068, r: 0.805

age-group ≥20 63–64. This sex distribution has been observed among adult cases in other countries, it is not due to a "Gregg effect" as it existed before Gregg's discovery in 1941 (9).

MATERIALS AND METHODS

Selection of subjects to be tested and collection of material. With reference to the epidemics recorded since 1925 thirteen locations were chosen for collection of material to be used in this study. From each part of the country the most central medical district was selected, where rubella had occurred most frequently and epidemics been most widespread. Five other districts were included as they were considered of interest, having in common that less than 20 cases in each district were reported during the epidemic '63–'64 (see Table 1). One location, Thingeyjarsýslur, is the rural medical district Breidumyrarhérad and the rural part of the adjacent Husavíkurhérad.

Only women were included in the series. The age of the women was in the range 5–49 years, divided into 12 age-groups. The age-groups chosen were composed of those born one year before and one year after each epidemic, beginning with '23–'26. Thus the oldest age-group (5–9 yrs.) and the youngest (1 yr.) that were subject to rubella for the first time in each epidemic are included. Because of the few inhabitants in some districts, some women slightly outside the chosen age-groups had to be included, but care was taken never to include women born during an epidemic.

In all areas except Reykjavík and Akureyri, the age-groups chosen included all women who could

TABLE 3. *Distribution of*

Year of birth	'64–'67	61 '63	55–59	52 '54	48–'51	44–'47
Age in years when tested in 1972	5–7	9–11	13–16	18–20	21 24	25 27
Number	189	178	206	153	121	104

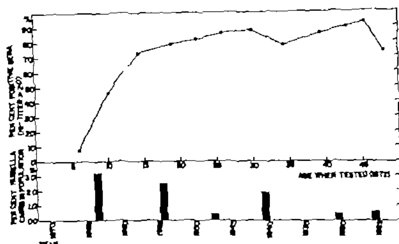


Fig. 3 Rubella HI antibody by age of subject. F value: 64 10 d.o.f. 11 and 1452, i.e. $p < 0.1$ per cent. The years and proportional sizes of the rubella epidemics since 1925 are shown below the scale applying to years is the same as abov In order that the epidemics experienced by each age-group can be seen by counting from right to left.

be traced. In Reykjavík, groups of 50 subjects were selected randomly from these age-groups, using municipal records. In Akureyri the same method was used to select from age-groups counting more than 50 females.

A standardized medical history was obtained from each subject and a blood sample collected. The blood samples were stored at +4 C before transport no more than 1–2 days after collection. The sera were then separated and stored t −20 C for 2–4 months before the test was done.

Information and blood samples were obtained from 1464 subjects. Distribution over age-groups is shown in Table 3. The number of subjects from each location can be seen from Fig. 5

Serological methods To assess the immunity the haemagglutination inhibiting antibodies (HI antibodies) were measured, using the Gothenburg modification of the Halonen method (4) The erythrocytes used are fresh, untreated pigeon blood cells, in a 0.5 per cent suspension. Commercial antigen which had titre of 1/128 was obtained from the Flow Laboratories. Each test included double serum controls which had a high and a low titre and a negative serum sample. Women with titre >20 were considered positive. The value 20 was regarded either as a false positive response or so low that it would not constitute a defence against infection. If a titre proved to be 20, the test was repeated.

RESULTS

The ratio of subjects that were seropositive in the HI-test (immunity ratio) is given in Fig 3 according to age-groups.

Girls born after the last epidemic ('63–64) proved to have an immunity ratio of 6.9 per cent. In 7 locations, however they were all negative, and the result does not warrant the conclusion that rubella is endemic in Iceland as an epidemic was beginning when the blood samples were collected. The next two age groups have gone through only one epidemic. Those in the older group had attained school age at the time of that epidemic. This age

ject A cording to Age

43	'35–40	'31–'34	'28–'30	'26–'27	'23–'25	Total
31	32 37	38–41	42–44	45–46	47–49	
26	110	99	73	59	42	1464

group has developed a higher immunity ratio than the younger group Difference was expected since children of school age communicate more than pre-school children.

Two of the age-groups, age 32–37 ys. and 47–49 ys., show a depression in the graph, but the difference from adjacent age-groups is not quite significant.

In each column depicted in Fig 4 two and two age-groups with the same relationship to different epidemics are taken together It can be seen that, in each case the epidemic to occur earliest in life contributes most to the immunity ratio in an age-group although relatively less when this epidemic was experienced at an early age.

The differences in titre according to age-groups *are that low titre values are rather* more frequent among those who experience their first epidemic at a young age as compared with age-groups in which children were of school age during the first epidemic, see Table 4 In the former age-groups the range of mean titre values is 107–126, being 115–148 in the latter

The difference in immunity ratio according to age-groups is found to be the same whether determined on the basis of the various locations or the entire series, p is always below 5 per cent, except in two rural districts (Kleppjárnsreykjah. and Reykhólah.) Age-groups that have been exposed to two epidemics have generally reached an immunity ratio of appr 80 per cent. In Reykhólahérad,

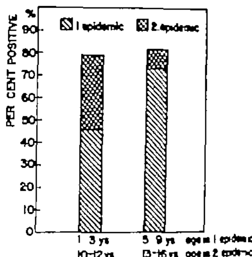


Fig 4 Probable rate of seroconversion according to age at the time of the first epidemic in lifetime. In the left column the immunity ratio of the age-group born 61–'63 is superimposed on that of the age-group born 52–54 the right column is constructed from immunity ratios of age-groups born 55–59 and 48–51 These agegroups were 1 3 and 5–9 ys. old at the time of the epidemics '54–55 and '63–'64

only few subjects are included in each age-group. There, subjects aged 18 ys. and above have an immunity ratio of 60 per cent. None of the epidemics is reported to have reached this district.

Fig 5 shows the immunity ratio in each location, the age-groups 16–42 ys. being shown separately There is some difference between rural and urban districts as can be seen from Table 5 Reykhólahérad clearly

TABLE 4 *Distribution of Titre Values According to Age of Subject at the Time of the First Epidemic in their Lifetime*

Titre	Age in 1 epidemic			Year of birth		
	1–4 ys.	5–9 ys.	Total	'61 '63	'55 '59	Total
40	141	110	251	25	33	58
80	170	189	359	22	69	91
160	123	138	261	25	36	61
320	37	49	86	9	11	20
640	8	15	23	1		5

The χ^2_{1} for the first two columns is 8.94 i.e. 5 per cent$<p<$10 per cent Results for the two age-groups that have only experienced one epidemic ('63–'64) are shown separately The χ^2_{1} for these two columns (omitting titre 640) is 8.18, i.e. 1 per cent$<p<$5 per cent.

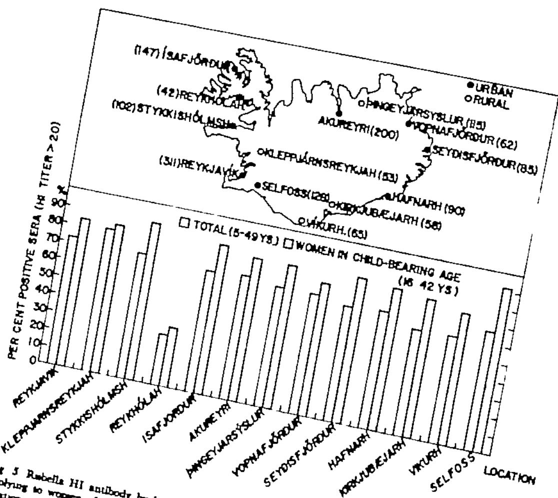


Fig. 5 Rubella HI antibody by location. F-value 3.61 d.o.f. 12 and 1445 i.e. $p<0.1$ per cent. Results applying to women of child-bearing age (16–42 yr.) are shown separately in the shaded columns. The locations are shown on the map above showing also whether they are urban or rural, and the number of subjects from each.

deviates from the rest. If compared with Table 1 it can be seen that the immunity ratio applying to the districts under study correlates moderately well with their epidemiological history with the exception of Kleppjarnsreykjahérad.

Table 6 shows the immunity ratio according to the medical history of the individual cases. Most of the subjects claimed to have caught the disease during the first or second epidemics to occur in their lifetime. The medical history was correct in 68.4 per cent of those who claimed to be certain. A negative medical history proved to be much less reliable than a positive one. Among the seropositive subjects, 52.1 per cent were aware of having had the disease.—An Irish study showed that 84 per cent of subjects giving a positive history were seropositive this ratio being 69 per cent for those with a negative history (8).

It has been assumed that 1 out of 10 to 20 rubella cases is reported (12, 13, 18). Using population statistics and the serological results, an estimate of the actual infection rate in the epidemic '63–'64 can be reached. In Dec. '63 23 422 Icelanders were 0–4 years old (i.e. born '59–'63), 22 140 were 5–9 yr. (born '54–'58). Among these, 46 per cent and 73 per cent, respectively, were infected ac

TABLE 5 *Rubella Immunity Ratio in Urban and Rural Districts*

Age in years when tested in 1972	Urban	Rural	Total
5–49	69.5 % (SE: 1 4 %)	62 1 % (SE 2 7 %)	67.8 % (SE: 1.2 %)
16–42	83.8 % (SE 1.3 %)	76.3 % (SE 2.8 %)	81 1 % (SE 1.2 %)

8 districts are urban, 5 are rural. Results applying to women of childbearing age (16–42 yr.) are shown separately (SE standard error)

TABLE 6 *Serological Results Related to History of Clinical Disease*

Reply to question concerning the medical history	Number	HI–result		Standard error
		Positive	Negative	
Yes	585	88.4 %	11.6 %	1.3 %
No	709	48.1 %	51.9 %	1.9 %
Unknown	170	79 4 %	20.6 %	3.1 %
Total	1464			

cording to our results obtained in age-groups born 61–63 and 55–59 see Fig 3 19 622 were 10–14 yr. (born 49–53) estimated infection rate 33 per cent (see Fig 4) 122 130 were 15 yr. and above, estimated infection rate 5 per cent. This adds up to 39 540 rubella patients, which is equal to appr 21 per cent of the population. Reported cases applied to 3.2 per cent of the population, i.e. roughly 1 out of 7 was reported.

DISCUSSION

Public Health records are the original source of information about epidemics in the country The records are accurate as to the time of occurrence of epidemics and nearly accurate as to their spread. Statistics on incidence are however very inaccurate, esp as regards rubella. The many reasons for this include uncertain diagnosis, harmlessness, foetuses excepted, and in some areas, absence of district doctors.

It is unlikely that rubella is endemic in Iceland now though Figs. 1 and 3 might seem to indicate that. Most of the cases reported in nonepidemic years were probably not correctly diagnosed, but there may have been introductions from abroad which did not cause epidemics (13) The immunity ratio of children born after the epidemic '63–'64 (6.9 per cent) is due to the beginning epidemic of 72–?

Iceland is a sparsely populated country (appr 200000 inh. now) and, until the last 15–20 years with poor communications esp in winter It is understandable that it takes a long time before a not very contagious disease like rubella may establish an epidemiological pattern in such a country and indeed, rubella epidemics do not yet show a quite regular pattern.

On the whole the immunity ratio obtained in this study is surprisingly high, and similar to that in many European and North-American countries, whereas immunity ratios are lower in many African and Asian countries (2)

Incidence being the least reliable piece of information, the interpretation of Fig 4 is difficult. There is undoubtedly some real increase in the proportional size of the epidemics. Rubella is reaching new districts probably in each epidemic esp. 40 41 and '63-

of Iceland. One of the authors is greatly indebted to Prof *Erik Lycke* and his staff at Kliniskt virologiska laboratoriet in Gothenburg, where he learned the technical details. We want to express our most sincere gratitude to *Stefán Adalsteinsson* Ph. D., for great help and advice on the statistical work. We also want to thank *Júlíus Sigurjónsson* professor of hygiene at the University of Iceland, for his suggestions.

REFERENCES

1 *Baldursson G Bjarnason Ó., Halldórsson S., Júlíusdóttir E. & Kjeld M.* Maternal rubella in Iceland 1963–1964 Some observations on 37 children with rubella syndrome. Scandinavian Audiology *Vol. 1 No 1 3–10 1972*

2. *Cockburn, W C.* World aspects of the epidemiology of rubella. Amer J Dis. Child. *118* 112–122, *1969*

3. *Gale J L., Detels R., Kim K S W., Beasley R. P & Grayston J T.* Epidemiology of rubella on Taiwan. Amer J Dis. Child. *118* 143–145, 1969

4 *Halonen P E., Ryan J M & Stewart J A* Rubella hemagglutinin prepared with alkaline extraction of virus grown in suspension culture of BHK-21 cells. (32038) Proc. Soc. Exp. Biol. Med. *125* 162–167 1967

5. *Hannesson G* Heilbrigdisskýrslur (Public health in Iceland) 1911–1920 Reykjavík *1922*.

6. *Hannesson G* Heilbrigdisskýrslur (Public health in Iceland) 1921–1925. Reykjavík 1927

7 Heilbrigdisskýrslur (Public health in Iceland from the years 1926–1970.

8. *Hillary I B.* Rubella in the republic of Ireland. Postgraduate Medical Journal, *July suppl. 1972* 18–21 1972.

9. *Ingalls T H., Babbott F L. Jr Hampson, K. W & Gordon J E.* Rubella: its epidemiology and teratology Am. J Med Sciences. 239 137/363–137/383 1960.

10 *Jónasson J* Skýrsla um heilbrigdi manna á Íslandi árid 1896. Ibid. from the years 1897–1900

11 *Sigurdsson B.* Medfætt heyrnarleysi af völdum raudra hunda—(Rilfregn) Læknablaðið *39* 73–75, 1955.

12. *Sigurjónsson, J* Medfætt heyrnarleysi og önnur vanheilindi af völdum raudra hunda. Læknablaðið *46* 63–71 1962.

13 *Sigurjónsson, J* Rubella and congenital deafness. Am. J Med. Sciences. *242:* 88/712 96/720 1961

14 *Sigurjónsson J* Skarlatssótt og raudir hundar á Íslandi á tímabilinu 1881–1900. Læknablaðið. *44* 183–188, 1960.

15 *Sveinsson K* Medfædd blinda og aðrar vanskapanir af völdum raudra hunda. Læknablaðið. *34* 147–154 1950.

16. *Tómasson B. (Ed)* Skýrslur um heilbrigði manna á Íslandi árin 1881–1890. Skrifstofa landlæknis, Reykjavík 1965.

17 *Tómasson, B. (Ed)* Skýrslur um heilbrigði manna á Íslandi árin 1891 1895 med viðauka við skýrslur árin 1896–1900. Skrifstofa landlæknis, Reykjavík, 1961

18. *Witte J J., Karchmer A W., Case G., Herrmann K I Abrutyn E., Kassanoff I & Neill J S* Epidemiology of rubella. Amer J Dis. Child. *118* 107–111 1969

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LIPOLYTIC ACTIVITY OF *STAPHYLOCOCCUS AUREUS* STRAINS FROM CASES OF HUMAN CHRONIC OSTEOMYELITIS AND OTHER INFECTIONS

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Hedström, S. A. Lipolytic activity of *Staphylococcus aureus* strain from cases of human chronic osteomyelitis and other infections. Acta path. microbiol. scand. Sect. B, 83 285–292, 1975

The fatty acid accumulation in a lipid emulsion broth was examined after growth of *S. aureus* strains incubated for 18 hours at 37 C. Among 40 strains from acute infections and from nasal carriers, Tween 80-positive staphylococci in phage group III caused accumulation of significantly larger amounts of fatty acids than did Tween 80-positive strains in phage group I and Tween 80-negative strains in phage groups I and III. Eight out of 17 *S. aureus* strains isolated from chronic osteomyelitis gave rise to low amount of, or total lack of fatty acids in the lipid broth. *Seven of these 8 strains were isolated from cases showing relapse after intensive* and prolonged antibiotic treatment. The lipid metabolism of 4 strains showing fatty acid accumulation in lipid broth, ranging from zero to the highest value found in the investigation, was further studied. Results of examination of lipolytic action on trioleinphospholipid and incorporation of ^{14}C-labelled oleic acid suggest that the degree of accumulation of fatty acids in lipid broth depends on variations in the lipolytic activity of the strains rather than on differences in incorporation of the produced fatty acids. The significance of fatty acid accumulation is discussed in relation to the prognosis of chronic staphylococcal osteomyelitis.

Key words: *Staphylococcus aureus*; lipolytic activity; chronic osteomyelitis; other infections.

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Osteomyelitis and furunculosis caused by *Staphylococcus aureus* often recur in spite of intense antibiotic treatment (12, 17). Various reasons have been proposed to explain the ability of the staphylococci to survive in the bone and bone marrow. Antibiotics are not considered to reach the bacteria in sclerosed and fibrous foci (9) and around sequestra (14). Staphylococcal L-forms are supposed to revert to ordinary bacterial forms, causing clinically manifest exacerbation of osteomyelitis (10, 16).

The focus of osteomyelitis as well as the focus of furunculosis has an abundance of lipids in its surroundings. *S. aureus* is known as one of the few pathogenic bacteria which produce enzymes with lipolytic activities (1, 19); therefore the interaction between lipids and staphylococcal strains isolated from cases of chronic osteomyelitis is of interest. This paper concerns the lipolytic activity of *S. au*

reus strains on triglycerides corresponding to those in human fat tissue and the significance of the fatty acids produced with special regard to strains isolated from chronic osteomyelitis.

MATERIALS AND METHODS

Bacteria

Seventeen strains of *S. aureus* were isolated from fistulae or the infectious foci at surgical procedures in human cases of chronic osteomyelitis. Some patients had relapses of osteomyelitis though they had undergone long-term treatment with antibiotics (12) while others remained healed throughout a long observation period after treatment. The strains were stored frozen in broth and were subcultured on nutrient agar before the investigation. The lipolytic activity of staphylococci has been shown to be stable after freezing and subculturing (2).

Forty strains of *S. aureus* belonging to phage groups I and III were collected from routine phage typing work (Department of Medical Microbiology, University of Lund, Lund). The strains were isolated from acute infections (septicaemia, wound infections, diarrhoea) and from nasal carriers. Phage-typing was performed, using the standard set of phages tested at RTD and 100 × RTD (5). At the same time, lipolytic activity was tested according to *Sierra* (18) using Tween 80 as substrate which is a water-soluble oleic ester of sorbitol anhydride.

Four strains were investigated with a view to lipolysis of triglycerides and incorporation of oleic acid: two (MJ, RP) were isolated from osteomyelitis which recurred in spite of intensive antibiotic treatment, one from a case of osteomyelitis (AN) without relapse after antibiotic treatment, and one (5605) from an acute infection.

Bacterial counts were made in a Petroff Hausser counting chamber under the phase contrast microscope.

Titrations of Fatty Acids in Lipid Broth after Growth of S. aureus

Lipolysis of a water-insoluble lipid substrate by all the staphylococcal strains was tested by titration of fatty acids accumulated during growth according to a method described by *Stewart* (19). As lipid substrate olive oil was chosen because its composition was similar to that of triglycerides in human fat tissue (4). The main triglyceride in olive oil and in human fat tissue is glyceroltrioleate. Sterile olive oil (Olive oil purum KEBO Sweden) was emulsified mechanically in casein-yeast-medium (CCY) (3) and the 1 per cent (v/v) emulsion was stabilized by 4 per cent (v/v) polyvinyl alcohol. Staphylococcal strains were inoculated into 3 ml of the lipid broth which was incubated for 18 hours at 37 C. The broth was extracted with 15 ml ether-acetone (equal parts) and 10 ml extract was titrated against 0.01 N alcoholic potassium hydroxide with thymolphthalein as indicator. As controls, uninoculated lipid broth and inoculated CCY containing only 4 per cent polyvinyl alcohol were examined in the same way.

Studies of Lipolytic Activity and Incorporation of Oleic Acid by Selected Strains of S. aureus

According to the method described above, only the accumulated fatty acids in the broth are measured. The presence or absence of accumulation depends on the balance between two possible processes: on the one side the lipolysis by bacteria, which results in production of fatty acids, and, on the other, the incorporation or utilization for oxidation of the fatty acids by growing bacteria. To discriminate between these two processes, 4 strains which caused large or no accumulation of fatty acids in broth were investigated further.

1. *Studies of lipolytic activity of S. aureus.* After growth of *S. aureus* for 18 hours at 37 C in rotating bottles containing 100 ml CCY-broth, the broth was sterile-filtered and examined for lipolytic action on water-soluble and water-insoluble lipid substrates according to the following methods using:

A. ^{14}C-triglyceride emulsion (stabilized with phospholipids) representing a water-insoluble lipase substrate. The release of labelled oleic acid was measured (15).
B. Micellar ^{3}H-monoglyceride solution, representing a solubilized esterase substrate. The release of labelled glycerol was measured (20).
C. Tributyrine emulsion, representing a short chain carboxylic ester substrate with and without the presence of 6 mM bile salts (sodium taurodeoxycholate). Free butyric acid was assessed by titration (8).

The lipid broth used for titration of fatty acid accumulation in broth, the nearest equivalent to A, possibly contaminated by partial glycerides though the oil used was of pure quality.

2. *Incorporation of oleic acid by S. aureus strains.* Suspensions of the *S. aureus* strains (~2 · 10[9] bacteria/ml) in two amounts, 0.1 and 0.9 ml each, plus culture medium (CCY-broth) to a total volume of 1.0 ml, were incubated with 0.5 ml of 0.2 mM ^{14}C-oleic acid complexed with bovine serum albumine (0.5 % w/v) in Hanks solution containing 20 mM HEPES pH 7.4 gassed with 95 % O_2/5 % CO_2. The incubation time was 1 hour at 37 C during rotatory shaking. After extraction,

TABLE 1 *Accumulation of Fatty Acids in Lipid Broth in Relation to Phage Group and Tween 80-reaction after Growth of S. aureus Strains from Acute I[...] ations and from Nasal Carriers*

Phage group Tween 80-reaction	I pos.	III pos.	I neg.	III neg.
	0.66	2.23	1 63	1.82
	1 18	1.31	0.03	1 74
	0.99	3.26	0.80	0.50
	0 93	3.35	0.71	0 86
	1.34	2.45	0.53	0.01
	0 52	1.65	0.22	0 49
	0 59	0.74	0.32	0.31
	0 89	1.25	0.81	0.66
	0.03	1 18	0.48	1 13
	1.23	1 19	0.14	1 12
Mean and standard deviation	0.85 ± 0.34	1.86 ± 0.87	0.37 ± 0.44	0.86 ± 0.56

* Strain 5605.
The figures denote ml acid corresponding to 0.01 N hydrochloric acid or × 10 μmol fatty acids accumulated in 2 ml of 1 per cent lipid medium during 18 hours of incubation.

the lipids were isolated by thin-layer chromatography on silicic acid with 4 per cent acetone in chloroform as solvent system. The spots were transferred to vials and counted by liquid scintillation spectrometry (21).

During the incorporation of C-oleic acid, the CO delivered from the bacteria was sampled and tested for $^{14}CO_2$ (21).

RESULTS

Grouping of the Staphylococcal Strains

The strains obtained during routine work were classified in phage groups I and III and according to Tween 80-reaction (Table 1). The osteomyelitis strains were grouped in two groups depending on whether or not relapse occurred after long-term antibiotic treatment. All strains isolated from cases of osteomyelitis were Tween 80-positive, 9 strains belonged to phage group I, one to phage group II, 6 to phage group III and one strain was not typable (Table 2).

Accumulation of Free Fatty Acids in Lipid Broth

The amount of fat-soluble acids accumulated during the growth of staphylococci in lipid broth varied from values corresponding to zero to more than 3 ml 0.01 N hydrochloric acid. The results were reproducible in repeated assessments after new inoculation and incubation. The amount of fatty acids accumulated by Tween-positive staphylococci in phage group III was significantly higher than that in other groups (Table 3). There was no significant difference between the other groups.

Very few of the routine strains gave low accumulation of fatty acids. Only 7 out of 40 strains gave fatty acids corresponding to less than 0.4 ml 0.01 N hydrochloric acid. Among the osteomyelitis strains, the frequency of strains giving a low accumulation was much higher. 8 out of 17 strains gave fatty acids corresponding to less than 0.4 ml 0.01 N hydrochloric acid (Table 2). Seven of these 8 strains were found in patients in whom osteomyelitis recurred after long-term treatment. In 2 patients (AW and NS) sequestra were found which seemed to be a probable explanation of the recurrence, but in the other cases there was no obvious cause for the exacerbation.

TABLE 2. *Accumulation of Fatty Acids in Lipid Broth after Growth of 17 S. aureus Strains from Cases of Chronic Osteomyelitis with and without Relapses after Antibiotic Treatment. All Strains Are Tween 80-positive*

Strains from relapses	Phage groups	ml 0.01 N acid	Strains from healed cases	Phage groups	ml 0.01 N acid
FP	III	0.30	RL	I	1.87
OP	I	0.21	GM	I	0.89
JL	I	0.04	AP	III	1.34
MJ	I	0.00	TP	I	1.38
AW	I	0.09	AJ	III	0.94
RP	III	0.01	ER	III	1.33
NS	I	2.32	GL	II	0.58
GJ	NT	0.31	AN	III	1.58
			JH	I	0.56

* See explanation under Table 1

TABLE 3. *Differences between Means (Table 1) Tested by Student's t-test*

Compared groups		t	P
III + and	I +	3.403	< 0.01
I —	III —	1.316	> 0.1
I +	I —	1.629	> 0.05
III +	III —	3.035	< 0.01
III +	I —	4.190	< 0.001

Roman numerals: Phage groups.
+ — Tween 80-reaction.

Study of Lipolytic Activity

The lipolytic activity of the broth filtered from 4 strains grown in CCY was tested by the methods A, B and C. Three strains were isolated from cases of osteomyelitis (MJ, RP and AN) and 5605 was a strain from an infection other than osteomyelitis, Tween 80-positive, and belonging to phage group III. This strain, of all strains examined, caused the largest accumulation of free fatty acids (3.35 ml 0.01 N acid, Table 1) according to the method of *Stewart* (19). The strain AN also caused accumulation of fatty acids in lipid broth, but the strains MJ and RP did *not*. The data obtained in the study of lipolytic activity according to the methods A, B and C are shown in Table 4.* It was shown that the strains AN and 5605, in relation to the number of bacteria, had a lipolytic ac- tivity on triglycerides as well as on mono-

TABLE 4. *Lipolytic Activity in the Filtered Supernatants of 4 S. aureus Strains Grown in CCY and Tested According to Methods A, B and C*

Method	Strains AN	MJ	RP	5603
A	1.18 10^{-3}*	0.54 10^{-3}	0.23 10^{-3}	0.37 10^{-3}
B	0.173	0.115	0.022	0.0?6
C with bile salt	1.5	1.8	not tested	n.t.
without bile salt	1.2*	1.7	n.t.	n.t.
Number of bacteria per ml broth before filtrated (× 10^{10}) ‖	5.8	5.5	2.6	2.7

* The figures indicate µmol fatty acid produced/min/ml supernatant.
‖ Counted in Petroff Hausser counting chamber

TABLE 5 *Incorporation of [1-^{14}C]-oleic Acid into Complex Lipids by Bacterial Preparations*

S aureus strain	Amount (ml)	^{14}C-oleate incorporated into non-polar lipids	intermediate fraction*	polar lipids*	sum
AN	0.1	1.83§	1.32	4.86	8.21
	0.9	3.33	3.63	11 47	18.43
MJ	0.1	0.92	0.54	1.89	3.35
	0.9	3.39	2.20	11 96	17 75
RP	0.1	1 14	0.50	3.05	4.69
	0.9	4 78	1 77	10.31	17.06
5605	0 1	1.04	0.80	3.39	5.23
	0.9	3.96	2.04	11.08	17.08

Non-polar intermediate, and polar lipids refer to areas with Rf's corresponding to triglycerides, di glycerides, and phospholipids, respectively

§ The numbers indicate per cent of added ^{14}C-radioactivity

glycerides higher than that of strains MJ and RP The lipolysis of tributyrine was rapid and any considerable difference between the strains MJ and AN was not seen.

Incorporation of Oleic Acid in Growing Staphylococci

The incorporation of ^{14}C-oleic acid by the 4 strains MJ RP AN and 5605 is summed up in Table 5 * During the incorporation, no strain produced any ^{14}C-labelled carbon dioxide indicating that the oleic acid was not oxidized and utilized as an energy source.

DISCUSSION

One of the aims of this investigation was to compare osteomyelitis *S aureus* strains with strains from other infections as regards the accumulation of fatty acids during growth of the staphylococci in a culture medium containing triglycerides.

Stewart (19) found an amount of 0.9 ml 0.01 N acid after growth of *S aureus* strains in a medium containing 1 per cent olive oil. On an average approximately the same amount of fatty acids was found in the present work, with two exceptions. The accumulation of fatty acids by Tween 80-positive phage group III strains was significantly higher and a large proportion of strains from osteomyelitis cases accumulated no, or a low amount of fatty acids during 18 hours of incubation in lipid broth. It is apparent that *the lipolysis of triglycerides in olive oil is not* related to the Tween 80-reaction (Table 1) The splitting of Tweens is supposed to be catalysed by an esterase, while fat-soluble substrates and phospholipids are hydrolysed by a lipase at an aqueous interface (1 19)

The second part of the investigation was a special study of 4 strains (MJ RP AN and 5605) concerning their lipolytic activity and utilization of fatty acids. The purpose was to determine why some strains caused an accumulation of fatty acids while other strains did not.

The rate of tributyrine lipolysis by the strains MJ and AN was high (Table 4) which is correspondent with previously reported results (19) The lipolytic activity tested on the water-insoluble trioleinphospholipid, of the strains MJ and RP was less than 50 per cent of the activity of the strains AN and 5605 in relation to the concentration of bacteria (Table 4) It was shown that the strains MJ RP AN and 5605 incorporated ^{14}C-oleic acid without any considerable differences (Table 5) and the ^{14}C-oleic acid was not utilized for oxidation. The balance be-

* With the kind assistance of Dr *P Belfrage* Dept. of Physiological Chemistry University of Lund, Lund, Sweden.

TABLE 6 *Relation between Lipolytic Activity and Incorporation of Oleic Acid in the Staph. aureus Strains AN, MJ, 5605 and RP*

Strain	Released amount of oleic acid (in μmol) per hour by supernatant corresponding to 10^{10} bacteria calculated from the figures in Table 4 A	Incorporation of ^{14}C-oleic acid (in μmol) per hour by 10^{10} bacteria calculated from the 0.9 ml values in Table 5	
		provided constant amount of bacteria during incorporation	provided maximal growth of bacteria during incorporation
AN	0.122	0.102	0.043
MJ	0.059	0.097	0.039
5605	0.127	0.093	0.037
RP	0.055	0.090	0.038

* Estimated by counting growing staphylococci in CCY-broth at 18 and 19 hours of incubation at 37° C.

tween the two processes lipolysis and utilization of produced fatty acids in a continued culture, determines whether or not an accumulation of fatty acids will result. The release of fatty acids from triglycerides by the strains MJ and RP was slower than the incorporation of fatty acids, while the strains AN and 5605 had a lipolytic activity (Table 6 Fig 1) higher than the rate of incorporation. Because the incubation of broths was interrupted at 18 hours, prior to the analysis of ^{14}C-oleic-acid incorporation, the growth of

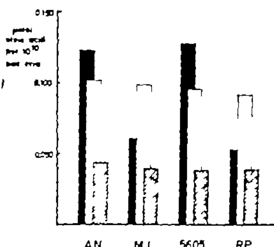


Fig 1 Diagram (from figures in Table 6 showing the relation between release of oleic acid from triglycerides (black columns) and the incorporation of ^{14}C-oleic acid at constant amount of bacteria (open columns) and maximal growth of bacteria (shaded columns)

staphylococci was probably not optimal during the period of incorporation. The incorporation during maximal and minimal growth is shown in Table 6 and Fig 1 the incorporation per 10^{10} bacteria is probably somewhere between the highest and lowest values shown. It is evident that the lipolysis by the strains AN and 5605 will result in an accumulation of fatty acids.

An accumulation of fatty acids may have inhibitory effects on staphylococci. Certain fatty acids exert a bacteriostatic or bactericidal effect on micrococci in a protein-free medium, whereas a stimulation effect of oleic, linoleic, and other fatty acids was seen in a medium containing albumine (6). *Kodicek & Worden* (13) had previously observed the inhibitory effect of oleic acid on grampositive bacteria but found no interference with growth in their study of short carbon-chained fatty acids. *Wynne & Foster* (22) demonstrated an inhibition of respiration of staphylococci by oleic and linoleic acids. Esters, triglycerides, and several oils have no significant toxicity for staphylococci (7).

The finding that there was no or only slight accumulation of free fatty acids in the medium after culturing certain osteomyelitis strains in lipid broth suggests that their production of fatty acids is not greater than the utilization. Most of these strains were isolated from cases resistant to intensive antibiotic therapy. Tween 80-positive staphylococci in

phage group III caused accumulation of a large amount of fatty acids in lipid broth and thus, there may be good possibilities for the inhibitory mechanisms mentioned above. Phage group III staphylococci were not found in cases of haematogenous chronic osteomyelitis (11) and were not as common as phage group I staphylococci in cases of relapsing osteomyelitis (12)

In the focus of osteomyelitis, *Lennert* (14) found that macrophages contained fagocytized droplets of fat. If a lipolysis occurs in such a focus, accumulated fatty acids which are not incorporated by bacteria may have an inhibitory effect on the growth of staphylococci and enhance the eradication of the micro-organisms. Such a mechanism would explain the preponderance in chronic osteomyelitis of staphylococcal strains which do not cause an accumulation of fatty acids. Staphylococci displaying a lipolytic activity in balance with the incorporation of released fatty acids may be more likely to survive in a focus containing lipids which can be utilized for the metabolism of the bacteria.

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REFERENCES

1 *Abramson C.:* Staphylococcal enzymes. In Cohen, J O. (Ed.) *The Staphylococci.* Wiley-Interscience, New York, 1972

2. *Alder V G., Gillespie W A Mitchell R. G & Rosendal K.:* The lipolytic activity of *Micrococcaceae* from human and animal sources. J Med. Microbiol. *6:* 147–154 1973

3. *Andersson S., Holme T & Wadström T.:* Influence of cultivation conditions on the production of extracellular proteins by *Staphylococcus aureus.* Acta path. microbiol. scand. Sect. B *79* 399–405 1971

4 *Belfrage P., Nilsson-Ehle P & Wiebe T.:* Effects on liver lipids and lipoprotein lipase activity of adipose tissue in relation to dietary lipids. Acta Med. Scand. Suppl. 532 1973.

5. *Blair J E. & Williams R. E. O.:* Phage typing of Staphylococci. Bull. Wld. Hlth. Org. *24* 771 784 1961

6. *Dubos R J* The effect of lipids and serum albumin on bacterial growth. J Exp. Med. *85:* 9 22, 1947

7 *Elek, D E.. Staphylococcus pyogenes* and its relation to disease. E. & S. Livingstone Ltd, Edinburgh and London, 1959 p. 501

8. *Erlanson C & Borgström B..* Tributyrine as a substrate for determination of lipase activity of pancreatic juice and small intestinal content. Scand. J Gastroenterol. *5* 293–295 1970.

9 *Fast H M., Villanueva A R. & Roth H..* Pyogenic osteomyelitis Diffusion in live and dead bone with particular reference to the tetracycline antibiotics. Henry Ford Hosp Med. Bull. *8* 255–262, 1960.

10 *Gordon S L., Greer R. B. & Craig C P.:* Recurrent osteomyelitis. Report of four cases culturing L-form variants of staphylococci. J Bone Joint Surg. 53 A 1150–1156, 1971

11 *Hedström S A & Kronvall, G.* Phage group, lipase activity and protein A content of *Staphylococcus aureus* strains from cases of chronic osteomyelitis. Scand. J Infect. Dis. *4:* 203–207 1972.

12. *Hedström S A..* The prognosis of chronic staphylococcal osteomyelitis after long-term antibiotic treatment. Scand. J Infect. Dis, *6* 33–38, 1974

13. *Kodicek E. & Worden, A N..* Effect of unsaturated fatty acids upon the growth of *Lactobacillus helveticus* and other gram-positive bacteria. Nature *154* 17–18, 1944

14 *Lennert K..* Pathologische Anatomie der Osteomyelitis. Z. Orthop. *100* (Suppl.): 27–64 1965

15 *Nilsson-Ehle P., Tornqvist H & Belfrage P..* Rapid determination of lipoprotein lipase activity in human adipose tissue. Clin. Chim. Acta *42* 383–390, 1972.

16. *Rosner R.:* Isolation of protoplasts of *Staphylococcus aureus* from a case of recurrent acute osteomyelitis. Am. J Clin. Path. *50* 385–390, 1968.

17 *Shelmire D S & Harwell, E. R.:* Staphylococcal skin infections. Treatment of impetigo and of acute and chronic furunculosis. Post grad. Med. *37* 202–206, 1965.

18. *Sierra G.:* A simple method for the detection of lipolytic activity of microorganisms and some observations on the influence of the contact between cells and fatty substrates. Antonie van Leeuwenhoek *23* 15–22, 1957

19 *Stewart G T:* The lipases and pigments of staphylococci. Ann. N Y Acad. Sci. *128* 157 151 1965.

20. *Tornqvist H Krabisch L. & Belfrage P..*

A simple assay for monoacylglycerol hydrolase activity of rat adipose tissue. J Lipid Res. *15* 291–294 1974

21 *Wiebe T & Belfrage P.*. Interaction between ethanol oxidation and fatty acid metabolism in isolated rat liver cells. To be published

22 *Wynne E. S & Foster J W.* Studies on the effects of C_{18}-unsaturated fatty acids on growth and respiration of *Micrococcus pyogenes var aureus.* J Infect. Dis. *86-87* 33–37 1950.

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BRIEF REPORTS

BORIC ACID TOLERANT *VIBRIO CHOLERAE*. BIOLOGICAL AND PHYSICAL PROPERTIES

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Ojo, M. O. Boric acid tolerant *Vibrio cholerae*: biological and physical properties. Acta path. microbiol. scand. Sect. B, *83:* 293–294. 1975.

Two boric acid tolerant variants were developed by passage in boric acid containing media from a mouse virulent parent *Vibrio cholerae* strain. The variants were stable, exhibited increased acid resistance, were less virulent to mice, but protected mice against challenge with the mouse virulent parent strain.

Key words: *Vibrio cholerae*; boric acid tolerance; biological and physical properties.

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The purpose of this work has been to develop boric acid tolerant strains of *Vibrio cholerae* and to study some of their biological and physical properties, acid tolerance and immunogenicity being the main problems in oral cholera vaccines.

Materials and Method

Development of boric acid tolerant strains. The parent *Vibrio cholerae* strain, serotype Inaba, strain 569BRPF* was passed in nutrient broth (Oxoid) containing increasing concentrations of boric acid to a maximum concentration of 0.5 per cent (w/v). Several culture lines were developed from this boric acid broth. The culture line leading to strain Ib5 was passed 20 times aerobically at 37° C in humid atmosphere on 0.5 per cent (w/v) boric acid nutrient agar and finally 10 times on 0.75 per cent and 1.0 per cent boric acid nutrient agar. Strain 18P2 was grown 18 times on 0.5 per cent and 10 times on 0.75 per cent boric acid nutrient agar.

Morphology and biochemical and serological test. Cell morphology was studied by a negative staining method using 3 per cent (w/v) Gurr's nigrosin W. S. Fermentation tests for glucose, maltose, sucrose, mannose, arabinose and lactose, as well as indole test and oxidase reaction, were carried out by conventional methods. Agglutination test was performed in polyvalent and monovalent *Vibrio cholerae* antisera (Difco).

Survival tests. Nutrient broth containing 10 per cent (v/v) phenol, 75 per cent (v/v) ethanol and 95 per cent ethanol, respectively, was prepared in addition to nutrient broth with pH adjusted to the following values: 2.0, 2.8, 3.0, 4.4 and 9.5. A loopful of culture mass grown on nutrient agar was suspended in tubes containing 3 ml of each of the nutrient broth preparations. Subcultures of a loopful from the suspensions were made after 1, 2, 5, 10, 15, 20 and 30 minutes and after 1, 2, 3, 4, 5, 10, 20, 30, 50, 70, 90 and 120 hours. The survival time is considered as the time of the last subculture with growth. In a similar way resistance to heat in nutrient broth at 56° C and 100° C was examined.

Virulence and protection tests. Eight-week-old White Swiss mice (male and female) weighing 16–18 g were used. Each mouse was inoculated intraperitoneally with 5.6×10^{8} viable organisms of the test strains in 0.5 ml of a 24-hour broth culture in sterile 5 per cent (w/v) hog gastric mucin (Koch-Light Laboratories, England). Control mice were likewise inoculated with 0.5 ml of sterile broth in mucin. The mice were observed for 72 hours and those surviving were kept for a further 14 days and challenged with 0.5 ml (4×10^{8}) viable organisms

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21 *Wiebe T & Belfrage P* Interaction between ethanol oxidation and fatty acid metabolism in isolated rat liver cells. To be published.

22. *Wynne E. S & Foster J W.*. Studies on the effects of C_{18}-unsaturated fatty acids on growth and respiration of *Micrococcus pyogenes var aureus*. J Infect. Dis. 86–87 33–37 1950.

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A NEW VIRUS SPECIFIED DETERMINANT OF HEPATITIS B SURFACE ANTIGEN

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Magnius, L., Kaplan, L., Vyas, G. N & Perkins, H. A. A new virus specified determinant of hepatitis B surface antigen. Acta path. microbiol. scand. Sect. B, 83 295–297 1975

One hundred and three samples from hepatitis B surface antigen (HB Ag) positive Californian blood donors were subtyped in immunodiffusion with respect to *d/y* *w/* and a_3 a^t Spur formation with a human antiserum indicated a previously unrecognized determinant *q* which was detected in 96 sera (4 *yw* 15 *a* *yw* 69 a_1 *dw* and 8 *ad*) Out of 8 sera which by spur formation were demonstrated to lack *q* 7 were further subtyped (2 a_1 *dw* and 5 a_3*dw*) Hence all 5 *dw* specimens were demonstrated to lack *q* thus indicating that *q* is specified by the virus. The possible relation of *q* to *x* with further implications for the specification of *x* is discussed.

Key words Hepatitis B surface antigen new virus specified determinant.

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The immunologic heterogeneity of hepatitis B surface antigen (HB_sAg) (1–2, 4 6–7 9 12–15) has been recognized by identification of a group specific determinant and tw pairs of "mutually exclusive determinants *d/y* (7) and *w/* (1) The determinant has been further subdivided (2, 14) to a_2 , and a_3 in order to explain aberrant spur formations and differences in the capacity for absorption of antisera by HB_sAg positive samples Until agreement has been reached upon a final nomenclature for the specificities associated with HB_sAg we here conform to the nomenclature of *Courouce-Panty & Soulier* (2, 14) which now most extensively takes into account the hitherto recognized diversity of HB_sAg. Here w report on the identification of determinant, tentatively designated *q* detected in study of subtypes of HB_sAg positive volunteer blood donors from California, utilizing human antiserum against HB_sAg which was found to form spurs in immunodiffusion tests between specimens containing *q* and *q* HB_sAg.

Material and Methods

The unabsorbed human antiserum (BA) against HB_sAg (previously used as anti-*d* after absorption with an a_3*yw*-antigen) was characterized in another report (10) Antisera against *y* *d* and *w* have previously been characterized (5 10 11) An antiserum against *t* and *d* was obtained by the immunization of a rabbit with purified a_1 *dw*-antigen according to procedures previously described (5) After absorption with a_3*yw*-antigen, reactivity towards a_3*t* and *w* was abolished. A rabbit antiserum against was kindly provided by Dr *G R.* Irwin (Washington, D C.) *Courouce-Panty* (Paris) kindly provided us with antisera against the and a_3-subdeterminants (Goat n.o 2 and Goat n.o 6 respectively) and with HB_sAg positive reference

Fig. 1. Absorption of antiserum (BA). Central well: A. Unconcentrated BA. B. One part BA four fold concentrated absorbed with one part a_2dwq. C. One part BA four fold concentrated absorbed with three parts a_2dwq. Peripheral wells: Four different HB$_s$Ag positive specimens with specificity as indicated in A also filled in corresponding positions in B and C.

sera with specificities as follows: 1) a_1yw 2) a_2yw 3) $a_3{}^4yw$ 4) a_4yw 5) $a_1{}^tdw$ 6) a_2dw 7) *ayr* and 8) *adr*. Immunodiffusion was performed as previously described (10).

Serum Specimens

One hundred and thirty-nine HB$_s$Ag positive samples were derived from the routine screening of volunteer blood donors by licensed counter-electrophoresis kits at the Irwin Memorial Blood Bank of San Francisco and at the Sacramento Blood Bank in northern California. Specimens were kept frozen until they were transported to Stockholm by air. Sodium azide was added to each specimen as a preservative.

Results

The results of gel diffusion analysis of 103 HB$_s$Ag positive specimens possible to subtype by immunodiffusion are depicted in Table 1. A previously unrecognized determinant was recognized by spur formation when the unabsorbed antiserum BA reacted with the standard a_2dw antigen and adjacent serum specimens. With this antiserum the standard antigen formed spurs over eight blood donor samples and total identity with 96 samples. Sera from the remaining 35 donors were too weak to give clear-cut results when tested with BA and are therefore not included in the following study. The new determinant was thus assumed to reside in the standard antigen and in 96 samples which gave a reaction of total identity with the standard antigen. After the absorption of BA with one serum of specificity a_2dw lacking the new determinant, this absorbed antiserum reacted with the 96 specimens believed to contain the new determinant, but failed to react with the eight which by spur formation were demonstrated to lack the new determinant. This has been tentatively designated as *q*. The gel diffusion analysis is depicted in Fig. 1. With the exception of the a_2dw specimen which was *q*-negative, the rest of the reference samples representing the different categories of HB$_s$Ag as described by *Courouce-Pauty & Soulier* (2, 14) were found to be *q*-positive.

TABLE 1. *Relation of the q-determinant to Subtypes of HB$_s$Ag in Californian Blood Donors. One q-negative Specimen Not Possible to Subtype According to Subdeterminants of a Was Excluded*

Category	Reactivity with absorbed anti-q		
	pos.	neg.	total
$a_1{}^1yw$	4	0	4
$a_1{}^tyw$	15	0	15
a_2dw	69	2	71
a_2dw	0	5	5
adr	8	0	8
Total	96	7	103

Discussion

In the past few years our knowledge of the antigenic heterogeneity of HB$_s$Ag has rapidly increased. Still we must be aware of the possible existence of additional distinct determinants and additional subdivisions of known determinants. Hence anomalous reactions on subtyping should be noted. In the present study subtyping of a series of HB$_s$Ag positive sera from northern Californian Blood Bank resulted in detection of eight samples which lacked a previously undetected determinant *q* otherwise present in 96 samples and in all HB$_s$Ag positive Swedish sera so far encountered. A reference sample containing a dw antigen was found to lack *q*. Interestingly all [illegible]

a_ydx-specimens encountered in this study were q-negative. The negative association of q to $_ydw$ was verified for 10 a_ydw-specimens which all lacked q according to tests with the antiserum BA carried out at Centre National de Transfusion Sanguine, Paris (3) Since a_ydw reflects a phenotype of the hepatitis B virus (HBV) the negativ association of q to a_ydw would be best explained by q being specified by HBV Whether the $a_y{}'dwq$ -specimens also reflect a new phenotype of HBV awaits further confirmation. The possibility that q might be masked or degraded in those samples has to be kept in mind. Since q thus seemed to be specified by the infectious agent it would appear unnecessary to compare q with x which was thought to be host derived. None the less, this comparison was made at two other laboratories. Dr *Le Bouvier* was unable to distinguish between q and x (8) but D *Couroucé Pauty* demonstrated a distinct difference between q and x (3) Since the original anti- has not been available to us, we hav been unable to further investigate the relationship of x to q However if the identity of q with x should prove correct, this study has demonstrated an association of x to the categories of HB_sAg as described by *Soulier & Couroucé-Pauty* (2, 12) suggesting that is specified by the viral genome.

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References 1 *Bancroft, W H M uden F K. & Russell, P K.*, J Immunol *109* 842–848 1972. —2. *Cou oucé-Pauty A. M & Soulier J P.*, Vox Sang. *27* 533–549 1974 —3. *Couroucé-Pauty A. M.*, Personal communication.—4 *Gust I D.*, Aust. J Exp. Biol. Med. Sci. *49* 625–627 1971 —5. *Iwarson, S Magnius, L., Lindblom A & Lundin P* Brit. Med. J *1*: 84–87 1973 —6. *Kim, C Y & Tilles J G.*, Infect. Dis. *123* 618–628, 1971 —7 *Le Bouvier G L.* J Infect. Dis. *123* 671–675 1971 —8. *Le Bouvier G L.* Personal communication.—9 *Levene C & Blumberg, B S.*, Nature (London) *221*: 195–196 1969 —10. *Magnius L. O & Espmark J A.*, J Immunol. *109* 1017–1021 1972.—11 *M gnius, L. O.*: Clin. Exp. Immun. *20* 209–216, 1975 —12. *Mazzur S Falker D & Blumberg, B. S* Nature (New Biol.) 239 89–91 1972.—13. *Renalo V K. London W T., Sutnick A. I Millman I & Blumberg, B S.*, Proc. Soc. Exp. Biol. Med. *134* 548–557 1970.—14 *Soulier J P & Couroucé-Pauty A M* Vox Sang *25* 212 234 1973 —15. *van Kooten Kok Doorschodt H J van der Akker R. & Gispen, R.*, Infect, Dis. *126* 117–122, 1972.

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MICROBIAL GROWTH ON AGAR SURFACES STUDIED BY INCIDENT LIGHT DIFFERENTIAL INTERFERENCE CONTRAST MICROSCOPY

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Froholm, L. O *Microbial growth on agar surfaces studied by incident light differential interference contrast microscopy* Acta path. microbiol. scand. Sect. B, 83 298–300 1975.

Microbial surface growth on routine opaque agar media was examined by various incident light microscopical techniques. Only differential interference contrast regularly gave good resolution and contrast. The arrangement of units approaching the size of individual bacteria may be judged by low power dry objectives.

Key words Microbial growth agar surfaces interference contrast microscopy

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Colony photography was necessary as a means of documentation in a study of agar corroding and spreading variants of *Moraxella* (2) Attempts to increase the magnification and improve the resolution led to experiments in which various microscopic techniques were used. The resulting discovery of the advantages of incident light differential interference contrast microscopy for the inspection of microcolonies on agar is briefly reported here

When the leading edge of a *Proteus mirabilis* swarm is studied images as those seen in Fig 1a and b will be obtainable under suitable growth conditions. There is a 20 sec. interval between the individual pictures. In Fig 1 lumps containing several cells are seen to move at a speed which initially was found to be approximately 3 μm/sec Part of the movement ceased during the experimental period, presumably due to drying heat, or the intense light In agreement with other studies (3) the most active motility is seen in the case of large oriented groups of cells, but also slender threadlike structures which may represent a single chain of cells are often seen to move rapidly A visible path is left on the agar after the bacteria have passed. The moving groups of bacteria may also be apparent in the solid cell layer within the colonies which is then rapidly changing its surface structure. This is demonstrated between triangles in Fig. 1

In other tests (not shown) polystyrene latex particles of 1.86 mμ diameter were resolved when spread on blood agar plates.

The object is seen as if it were illuminated by oblique light, giving the impression of a relief image. The interference contrast adapter has to be adjusted to a grey colour to give the best resolution. It is also important to be able to rotate the object and select the best position in terms of resolution and contrast. An intense and well adjusted source of light giving a bright image is essential.

Incident light differential interference microscopy seems to offer very good views of early microbial surface growth and the purpose of this communication is to make it better known. The following explanations are intended to indicate the possibilities and the limitations of the method and should not be considered a complete treatment of the subject.

Differential interference contrast is dependent upon rays of polarized light being divided and ...

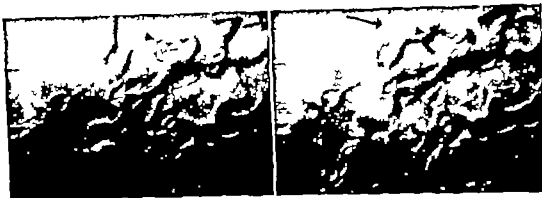


Fig. 1. Differential interference contrast microscopy of *Proteus mirabilis*. A Zeiss Universal microscope with the vertical illuminator IIIC, a H PL POL reflector polarizer analyzer and a 60 W lamphouse was adjusted according t the manufacturer's instructions and used with the objective EPIPLAN 16× N.A. 0.35 POL and the corresponding interference contrast dapter *P mi abilis* B 13678/72 from a spreading zone was inoculated as a spot on a human blood agar plate and incubated in a very humid atmosphere (2) until the appearance of swarming. It was a 20 sec. interval between a and b. A faint mark left on the agar after passage of the bacteria is indicated with an arrow and between triangles is a region with colony top surface changes during the experimental period. 155 × *P mirabilis* B 13678/72 was strain isolated in the diagnostic laboratory at this Institute and obtained through the courtesy of Dr *Kristian Odegaard*

lowing slightly different paths through the object, again reconstituted and allowed to interfere before reaching the eye (1 4 6 8 9). The optical system must include a polarizer and an analyzer. Special polarized light objectives with interference contrast adapters, containing a double prism, are required for work with incident light according to the Nomarsky system used here. Local differences in optical paths re made visible (9). Apparent dif ferences in height may be due merely to phase differences (6).

Initial investigations made it clear that a long, free distance between the front of the objective lens and the object under study was required. This is of course important with a view to facilitating operation and reducing the danger of touching the agar surface with the bacterial growth, but apparently it is also important for optical reasons. It was quite impossible to use the regular EPIPLAN 40× 0.85 N.A., POL objective (work ng distance 0.23 mm) while LD-EPIPLAN 40× 0.60 N.A., POL (working distance 2.3 mm) could be used with some success and definitely increased resolution as compared to EPIPLAN 16× 0.35 N.A. POL (working distance 2.7 mm). It was, however often difficult to obtain good contrast with the LD 40× objective and troublesome to use it routinely. The problem invol ed in loss of contrast t short working distances is believed to be related either to the higher numerical aperture or to depolarization of light at oblique incidence between the object and the front lens of the objective. Depolarization mechanisms are also thought to be responsible for the loss of contrast occasionally experienced during investigation of objects on clear agar media where the light reaches the under surface of the gar as well as the Petri dish and, in addition, is distributed widely in the agar itself. Immersion systems were avoided be cause of contamination problems and possible distorting influences on the colonial morphology.

Results obtained by other incident light microscopical techniques are shown in Figs. 2 and 3 which display an old agar plate with a spreading zone of *Morax lla nonliquefaciens* NCTC 7784 SO-a (2) and an 18 hour culture of *Moraxella lingua* A 1702, respectively. See the legend for further details. In Figs. 2a and 3a, typical images obtained by differential interference contrast are shown, in 2b and 3b those obtained by polarized light using the same objects without the inter ference contrast device and the analyzer and in 2c and 3c those obtained if an ordinary bright field incident light objective is used. It is seen that differential interference contrast is of particular value in the situation shown in Fig. 3 where the other methods were almost useless. In the other case, the advantages are less apparent—the resolution is about the same and also seemingly the contrast.

Comparison with known methods of agar plate microscopy shows that a similar relief image is obtained by oblique light transmission microscopy (5 7) which requires clear agar media and in certain directions suffers from reduced resolution. Phase contrast microscopy of lear agar

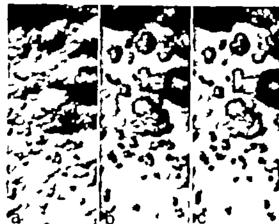


Fig 2 Comparison of incident light microscopy images. *Moraxella nonliquefaciens* NCTC 7784 SC-a (2) was adapted to growth on blood agar supplemented with 0 05 per cent sodium dodecyl sulfate and grown for 48 h at 33 C in a humid atmosphere. The plate was left in the laboratory for 5 days before microscopy A zone of spreading growth is seen. Zeiss Photomicroscope III, the light source was a HBO 50 lamp conditions otherwise as in the legend to Fig. 1 In a) EPIPLAN 16× 0.35 N.A. POL and corresponding interference contrast device in b) the same objective without the latter device and also the analyzer removed from the light path, further in c) the polarizer was replaced with a grey filter with 3 per cent transmission and the objective HD Achromat 16× 0.30 N.A. was used in the bright field mode. 315×

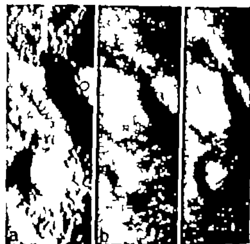


Fig 3 *Moraxella kingae* A 1702 (see 2 for ref.) mixture of spreading corroding ( ) and non-spreading and non-corroding (O) colonies after 18 h growth on blood agar at 33 C in a humid atmosphere. Details of a, b and c as in *Fig. 2* 315×

plate cultures is possible if a long focal distance condensor and dry objectives are used (3) This is an excellent method, but occasionally problems are encountered, for instance scattering of light in the agar resulting in suboptimal phase contrast conditions. Phase contrast does not usually give the three-dimensional image or show the colony surface but outlines very clearly individual cells at the edges of colonial growth.

In conclusion, the present method is proposed as an aid in the inspection of early microbial growth on routine opaque agar plates whenever the morphology of unit close to the size of individual bacteria may be of interest. It is less well-suited for inspection of bigger colonies and sometimes difficulties with clear agar media may be involved when the present equipment is used. Previous methods of incident light microscopy in microbiology (10 11) are extended.

References 1 *Allen R. D David G B & Nomarski G.*, Z. wiss. Mikr 69 193-211 1969 — ... *Bøvre K. & Frøholm L. O* Acta path. microbiol. scand. Sect B 80 629-640, 1972.—3. *Henrichsen J* Bact. Rev 36 478 503, 1972 —4 *Hoffmann R. & G ss L.*, J microscopy 91 149-172, 1970 —5. *Jephcott A E. & Reyn, A* Acta path. microbiol. scand Sect. B, 79 609-614 1971 —6. *Lang, W* Zeiss Informat. 71 12 26, 1969 — 7 *Mu las J M & Ales J M* Appl. Microbiol. 25 484-488, 1973 —8. *Neupert H* Zeiss Informat. 65 96-97 1967 —9 *Padawer J* J. roy. micr Soc. 88 305 349 1968.—10. *Paton A M & Jones S M* J Appl. Bact 36 441 443, 1973 —11 *Pearce T W & Powell E O* J gen. Microbiol. 5 91 103 1951

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LINCOMYCIN IN SELECTIVE MEDIUM FOR THE ISOLATION OF *NEISSERIA GONORRHOEAE*

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Ødegaard, K., Solberg, O., Lind, J., Myhre, G. & Nyland, B. Lincomycin in selective medium for the isolation of *Neisseria gonorrhoeae*. Acta path. microbiol. scand. Sect. B, 83: 301–304, 1975.

For the isolation of gonococci the selective culture medium containing colistin, vancomycin, nystatin and trimethoprim which is usually employed has been changed by substituting lincomycin for vancomycin. The best result was obtained if a concentration of ½ µg lincomycin/ml medium was used. This is a concentration of lincomycin considerably lower than that which by other investigators is considered most suitable for the purpose. However, the culture medium used by the latter did not contain trimethoprim. The use of ½ µg lincomycin/ml instead of vancomycin 3 µg/ml in the medium caused a slightly more pronounced growth of unwanted organisms. In spite of this, the results obtained by the medium containing lincomycin showed that the number of samples positive for gonococci was 7 per cent higher, and that the number of patients with gonococcal infections to be discovered was 4 per cent higher than the numbers obtained by the medium containing vancomycin. The results were considered highly favourable and, accordingly, by now our laboratory uses ½ µg lincomycin/ml medium in the routine isolation of gonococci.

Key words: *Neisseria gonorrhoeae*; isolation; lincomycin; selective medium.

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In the laboratory for bacteriological diagnosis of gonococcal infections, we have for some years used a selective culture medium for the cultivation of gonococci, employing colistin, vancomycin and nystatin as inhibitors of contaminating growth as recommended by *Thayer & Martin* (11). In addition, we have since 1970 used trimethoprim lactate (9, 10, 12) at a concentration of 5 µg/ml medium (12). This has improved our results to such an extent that we are now able to isolate gonococci from the rectum in more than 50 per cent of women with bacteriologically diagnosed gonorrhoea (13).

However, vancomycin included in the medium may inhibit the growth of some gonococcal strains (1, 8), and *Brorson et al.* (2) who substituted lincomycin for vancomycin in their medium for isolation of gonococci, obtained a net gain of about 10 per cent of bacteriologically diagnosed cases of gonorrhoea. Their medium did not contain trimethoprim and, the aim of this paper is to present the results of a comparison of cultivation of *N. gonorrhoeae* on our routine medium containing colistin, vancomycin, nystatin and trimethoprim and on a medium of the same composition except that vancomycin is replaced by lincomycin.

Material and Methods

The material consisted of specimens sent to the laboratory during the year 1974 for routine cultivation of gonococci. The specimens were obtained from patients examined at the Oslo Bureau

Fig 2 Comparison of incident light microscopy images. *Moraxella nonliquefaciens* NCTC 7784 SC-a (2) was adapted to growth on blood agar supplemented with 0.05 per cent sodium dodecyl sulfate and grown for 48 h at 33 C in a humid atmosphere. The plate was left in the laboratory for 5 days before microscopy A zone of spreading growth is seen. Zeiss Photomicroscope III the light source was a HBO 50 lamp, conditions otherwise as in the legend to Fig. 1 In a) EPIPLAN 16× 0.35 N.A. POL and corresponding interference contrast device, in b) the same objective without the latter device and also the analyzer removed from the light path, further in c) the polarizer was replaced with a grey filter with 3 per cent transmission and the objective HD Achromat 16× 0.30 N.A was used in the bright field mode. 315×

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In conclusion, the present method is proposed as an aid in the inspection of early microbial growth on routine opaque agar plates whenever the morphology of units close to the size of individual bacteria may be of interest. It is less well-suited for inspection of bigger colonies and sometimes difficulties with clear agar media may be involved when the present equipment is used. Previous methods of incident light microscopy in microbiology (10 11) are extended.

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ELECTRON MICROSCOPY OF *TRICHOMONAS VAGINALIS DONNÉ* INTERACTION WITH VAGINAL EPITHELIUM IN HUMAN TRICHOMONIASIS

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Nielsen, M. H. & Nielsen, R. Electron microscopy of *Trichomonas vaginalis Donné*. Interaction with vaginal epithelium in human trichomoniasis. Acta path. microbiol. scand. Sect. B, 83: 305–320, 1975.

Light and electron microscopic examination of portio biopsies from eleven patients with trichomoniasis vaginalis revealed in four patients clusters of cells of *T. vaginalis* (*T. vag.*) which were attached to the vaginal mucosa. The minimum gap between adjacent trichomonad cells was the size of gap junctions (2 nm). Cells of *T. vag.* invaded superficially located epithelial cells but did not penetrate to the deeper cell layers of the epithelium. The latter, however, were frequently infiltrated with neutrophilic granulocytes. Contact between cells of *T. vag.* and neutrophils was not observed. Trichomonads which were attached to epithelial cells contained a dense network of cytoplasmic microfilaments in the part of the cell which came into contact with the epithelium. The remaining part contained the organelles normally seen in *T. vag.* Endocytotic cell activity of amoeboid *T. vag.* occurred from the free cell surface only. A cell coat on the cell membrane—formed by bristles—was confined mainly to pinocytotic invaginations. Glycogen granules which were absent from the larger part of the epithelium were densely packed in the trichomonad cells. The findings in this study indicate that the interaction between the cells of *T. vag.* and the vaginal epithelium takes place primarily at a distance, probably by means of substances released into the vaginal fluid, and secondly by a direct cell contact mechanism.

Key words: *Trichomonas vaginalis*; Vaginitis; Parasites; Protozoa.

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For many years *Trichomonas vaginalis Donné* (*T. vag.*) has been associated with disorders of the female genital tract. Infection by the organism may give symptomatic vaginitis, urethritis and prostatitis, but the disease may remain in a latent phase. The symptoms are discharge, itching, pain and dysuria. The vaginal discharge is irritating and frothy, often with an offensive odour. On inspection, the vaginal mucosa may be affected with pronounced hyperaemia and minute areas of haemorrhage. The *T. vag.* infection may give rise to abnormal vaginal

dium. However we considered the results obtained by the medium containing ½ µg lincomycin/ml to be highly favourable and, by now it is used routinely in this laboratory for the isolation of gonococci.

According to our opinion, the combination of lincomycin and trimethoprim might be the reason why we arrived at a suitable concentration of lincomycin much lower than that reported by other authors. There is a possibility that lincomycin and trimethoprim act synergistically on the gonococci. Using the methods of *Møller & Holmgren* (5) we were not able to demonstrate any synergistic action between the two antibiotics which might inhibit gonococcal growth. However if the sensitivity discs (Biodisk AB, Stockholm)—the lincomycin disc containing 15 µg and the trimethoprim disc 1.2 µg—were placed at a distance of about 3 mm from each other on a nonselective culture medium inoculated with gonococci, the trimethoprim disc caused on some gonococcal strains an increase of the inhibition zone produced by the lincomycin disc on the gonococcal growth the trimethoprim disc itself failed mainly to produce a growth inhibition zone at all (Fig. 1) This may be interpreted as a possible enhancement of the inhibiting effect of the lincomycin.

Using lincomycin as one of the inhibitors of unwanted bacterial growth has a drawback in one respect as compared with vancomycin in that lincomycin does not inhibit enterococci (6) Most enterococci are, however highly sensitive to trimethoprim (4) thus, the combined use of lincomycin and trimethoprim in the medium is of advantage in this respect.

We are indebted to *Arne Høeby* M.D. for the photography

References 1 *Berger U.*: Z. Med. Microbiol. Immunol. *152* 169–172, 1966.—2. *Brorson J. E., Holmberg, I., Nygren B. & S eberg, S* Brit. J vener Dis. *49* 452–453 1973.—3. *Cross R. C Hager M B., Neibauer R., Pasternack B. & Brady, F J* HSMHA Hlth. Rep. *86* 990–992, 1971.—4. *Darrell J H., Garrod L. P & Waterworth, P M* J clin. Path. *21* 202–209 1968.—5. *Møller O & Holmgren J* Acta path. microbiol. scand. 76 141–145 1969.—6 *Potuznik V & Hauer O* J Hyg Epidemiol. Microbiol. Immunol. (Praha *13* 366–373 1969.—7 *Rey A* Bull. Wld Hlth Org. *40* 245–255 1969.—8. *Reyn A & Bentzon, M W.*. Brit. J vener Dis. *48* 363–368, 1972.—9. *Riddell, R. H & Buck A C.* J. Clin. Path. *23* 481–483 1970.—10. *Seth A.* Brit. J vener. Dis. *46* 201–202 1970.—11 *Thayer L. D & Martin, J E., jr.* Publ. Hlth Rep. *81* 559 562, 1966.—12. *Ødegaard K.*, Acta path. microbiol. scand. Sect. B, *79*: 545–548, 1971.—13 *Ødegaard K* Acta Dermatovener (Stockholm) *52* 326–332, 1972.

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Nielsen, M. H. & Nielsen, R. Electron microscopy of *Trichomonas vaginalis Donné*. Interaction with vaginal epithelium in human trichomoniasis. Acta path. microbiol. scand. Sect. B 83: 305–320, 1975.

Light and electron microscopic examination of portio biopsies from eleven patients with trichomoniasis vaginalis revealed in four patients clusters of cells of *T. vaginalis* (*T. vag.*) which were attached to the vaginal mucosa. The minimum gap between adjacent trichomonad cells was the size of gap junctions (2 nm). Cells of *T. vag.* invaded superficially located epithelial cells but did not penetrate to the deeper cell layers of the epithelium. The latter, however, were frequently infiltrated with neutrophilic granulocytes. Contact between cells of *T. vag.* and neutrophils was not observed. Trichomonads which were attached to epithelial cells contained a dense network of cytoplasmic microfilaments in the part of the cell which came into contact with the epithelium. The remaining part contained the organelles nomally seen in *T. vag.* Endocytotic cell activity of amoeboid *T. vag.* occurred from the free cell surface only. A cell coat on the cell membrane—formed by bristles—was confined mainly to pinocytotic invaginations. Glycogen granules which were absent from the larger part of the epithelium were densely packed in the trichomonad cells. The findings in this study indicate that the interaction between the cells of *T. vag.* and the vaginal epithelium takes place primarily at a distance, probably by means of substances released into the vaginal fluid, and secondly by direct cell contact mechanisms.

Key words: *Trichomonas vaginalis*; Vaginitis; Parasites; Protozoa.

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For many years *Trichomonas vaginalis Donné* (*T. vag.*) has been associated with disorders of the female genital tract. Infection by the organism may give symptomatic vaginitis, urethritis and prostatitis, but the disease may remain in a latent phase. The symptoms are discharge, itching, pain and dysuria. The vaginal discharge is irritating and frothy, often with an offensive odour. On inspection, the vaginal mucosa may be affected with pronounced hyperaemia and minute areas of haemorrhage. The *T. vag.* infection may give rise to abnormal vaginal

dium. However we considered the results obtained by the medium containing ½ μg lincomycin/ml to be highly favourable and, by now it is used routinely in this laboratory for the isolation of gonococci.

According to our opinion, the combination of lincomycin and trimethoprim might be the reason why we arrived at a suitable concentration of lincomycin much lower than that reported by other authors. There is a possibility that lincomycin and trimethoprim act synergistically on the gonococci. Using the methods of *Møller & Holmgren* (5) we were not able to demonstrate any synergistic action between the two antibiotics which might inhibit gonococcal growth. However if the sensitivity discs (Biodisk AB, Stockholm)—the lincomycin disc containing 15 μg and the trimethoprim disc 1.2 μg—were placed at a distance of about 3 mm from each other on a nonselective culture medium inoculated with gonococci, the trimethoprim disc caused on some gonococcal strains an increase of the inhibition zone produced by the lincomycin disc on the gonococcal growth the trimethoprim disc itself failed mainly to produce a growth inhibition zone at all (Fig 1) This may be interpreted as a possible enhancement of the inhibiting effect of the lincomycin.

Using lincomycin as one of the inhibitors of unwanted bacterial growth has a drawback in one respect as compared with vancomycin in that lincomycin does not inhibit enterococci (6) Most enterococci are, however highly sensitive to trimethoprim (4) thus, the combined use of lincomycin and trimethoprim in the medium is of advantage in this respect.

We are indebted to *Arne Holby* M.D. for the photography

References 1 *Berger U.*. Z. Med. Microbiol. Immunol. *152* 169–172, 1966.—2. *Brorson J Holmberg, I., Nygren B. & Seeberg, S* Brit. J. vener Dis. *49* 452–453 1973.—3. *Cross, R. C., Hoger M B. Neibauer R. Pasternack, B. & Brady F J* HSMHA Hlth. Rep. *86* 990–992, 1971.—4. *Darrell, J H., Garrod L. P & Waterworth, P M* J clin. Path. *21* 202–209, 1968.—5. *Møller O & Holmgren J.:* Acta path. microbiol. scand. *76* 141–145 1969.—6 *Potuznik, V & Hauer O* J Hyg. Epidemiol. Microbiol. Immunol. (Praha). *13* 366–373 1969.—7 *Reyn A* Bull. Wld. Hlth. Org. *40* 245–255 1969.—8. *Reyn, A & Korner, M W* Brit. J vener Dis. *48* 363–368, 1972.—9 *Riddell, R. H & Buck A C* J Clin. Path. *23* 481–483 1970.—10. *Seth A* Brit. J vener. Dis. *46* 201–202, 1970.—11 *Thayer L. D & Martin J E., Jr.*. Publ. Hlth. Rep. *81*: 559 562, 1966.—12. *Odegaard K* Acta path. microbiol. scand. Sect. B *79* 545–548, 1971.—13. *Odegaard, K.* Acta Dermatovener (Stockholm) *52* 326–328, 1972.

RESULTS

Vaginal cytology The vaginal smears showed no atypical epithelial cells or tumour-suspect cells. Routine cytological examination revealed *T vag* in smears from three patients only (Nos. 450 451 551 Table 1)

Vaginal biopsy In all patients, the portio biopsies showed chronic unspecific inflammation with subepithelial accumulation of plasma cells lymphocytes and neutrophilic granulocytes. In three patients (Nos. 450, 452 and 554 Table 1) showing a cervical erosion, the epithelial lining was a single layer of columnar epithelium, in the other eight patients it was stratified squamous epithelium. The lumen of the short glands was packed with neutrophils.

The stratified squamous epithelium from the eight patients showed a heterogeneous histological picture. A common finding, however was slight hyperplasia. In case of slight inflammation, neutrophils were confined to the most superficial part of the epithelium (Fig 1 b) whereas in more severe stages, neutrophils were found in the deeper layers also. The latter findings were often combined with ulceration of the surface and a certain degree of uniform atypia in which all layers of the cervical epithelium showed hyperchromasia and nuclear enlargement. The neutrophils located near the surface of the stratified squamous epithelium (NG Figs. 4 5) were placed in large lacunae. Apart from neutrophils and occasional lymphocytes, the content of these lacunae was almost unstructured (GR, Fig. 4) A minor part of the epithelium contained glycogen (Fig. 1 a) the areas with and without glycogen being sharply segregated. Only the areas without glycogen were normally infiltrated by neutrophils. Minor clusters of *T vag* cells were occasionally found on the surface of the stratified squamous epithelium, whereas they were always absent on the columnar epithelial surface of erosions. The areas with trichomonads often appeared as shallow depressions on the epithelial surface.

In seven patients (three with cervical erosions) no *T vag* cells were found attached to the epithelium. Clusters of *T vag* were seen on epithelium with and without cells containing glycogen.

Electron microscopy Clusters of trichomonad cells which were located on the epithelium consisted of free cells and cells which were attached to epithelial cells (Fig. 2, 3) In the large clusters the trichomonad cells were often densely packed forming a co-

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Figs. 1 and 1 b 1 μm sections. Cells of *T vag* (AT) attached to stratified squamous epithelium with glycogen containing cells (Fig. 1 a) and without glycogen containing cells (Fig. 1 b) Neutrophils (NG) infiltrate the latter only Magn.: 400 ×

Fig 2 Two amoeboid cells of *T vag* attached to the epithelium. Part of the mastigont is visible capitulum of the axostyle (A) and basal bodies/flagella (K) Both cells have undulating membrane (UM) and costa (C) located at the free cell surface. EC denotes the ectoplasm. Phagosome (PL) with mycoplasms and chromatic granules (CG) are visible. Debris of trichomonad cells is marked DB. The stratified squamous epithelium with intracellular glycogen (GL) shows no evidence of leucocytic infiltration. Magn. 5400 ×

Fig 3 Part of *T vag* cluster on stratified squamous epithelium. Epithelial cell (EP) with glycogen granules (GL) and amoeboid *T vag* (AT) on the surface. The ectoplasm (EC) in amoeboid trichomonads is confined to the part of the cell which is in contact with the epithelium. Non-amoeboid *T vag.* cells (NT) have no ectoplasm. Phagosomes with ingested mycoplasms are marked PL. N indicates the nucleus. CG the chromatic granules, and DE part of desquamated epithelial cell. Magn. 6800 ×

Fig 4 Stratified squamous epithelium (EP) infiltrated by neutrophilic granulocytes (NG) The neutrophilic granulocytes are transformed by lysis to a homogeneously stained material (GR) filling the intercellular space. Basal bodies/flagella (K) are visible on one of the trichomonad cells attached to the epithelium. Magn. 8300 ×

Fig 5 Surface layer of inflamed epithelium. The intercellular lacunae show lysed or partly lysed neutrophilic granulocytes (NG) a single lymphocyte (L) and the flocculent material which gains access to the surface (arrow) Epithelial cells are marked EP and partly lysed neutrophils in the vaginal fluid (DB) Magn. 4500 ×

cytology which occasionally leads to a suspicion of malignancy However no direct causal relationship between *T vag* and cervical carcinoma has ever been proven (4 5 26)

All clinical evidence of trichomoniasis disappears after peroral treatment with metronidazole which eliminates the trichomonads selectively (10 41) This fact as well as previous clinical and experimental evidence (44 19) indicate that *T vag* is truly pathogenic for the human host. However almost no information is available concerning the mechanism by which the parasite causes pathological changes in human subjects. Experimental infection of laboratory animals (7 15 21) and tissue cultures (8, 12 20 27) have demonstrated a cytopathogenic effect of *T vag* In tissue cultures, it seems to depend on an "intimate contact" between trichomonad and tissue culture cells, and, to a lesser degree, on the effect of toxins in the culture medium (8, 12, 27) However as demonstrated recently (7) in experimentally infected mice, the host cells are damaged before they come into contact with trichomonad cells. It is mysterious how *in vivo* cells of *T vag* causes epithelial lesions in the human vaginal mucosa. Therefore, the aim of the present study was to search, at the fine structual level, for a possible interaction between the trichomonad cells and the stratified squamous epithelium of portio vaginalis.

MATERIAL AND METHODS

Portio biopsies were removed by biopsy forceps from 11 patients aged 18 to 26 years who attended the Outpatients Clinic of the Department of Dermatology and Venerology Rigshospitalet, Copenhagen (Table 1) To varying extents, the patients complained of increased vaginal discharge and itching. On vaginal inspection, a yellow or greenish, purulent, foamy vaginal secretion was demonstrated. In ten patients the cervix had "strawberry" appearance. In all cases, *T vag* was demonstrated by direct microscopy of vaginal fluid in wet preparation and by cultivation from vaginal swabs, as described previously (36) The trichomoniasis was ultimately cured by metronidazole treatment (200 mg three times daily for 7 days) of patients as well as their sexual partners. In addition to trichomoniasis, four patients had genital gonorrhea which was treated with probenicid tablets 1 g and aqueous benzyl penicillin 5 million I.u. (37)

Vaginal smears were prepared according to *Papanicolaou* (38) for cytological examination.

The biopsies were divided into two pieces, one which was fixed in 4 per cent formaldehyde and prepared for routine histological examination, and one which was fixed in Karnowsky's fixative followed by osmium tetroxyde and prepared for electron microscopy as previously described (32) Electron microscopy was performed by Siemens Elmiskope 1A electron microscope.

TABLE 1 *Clinical and Histological Data Concerning the 11 Patients with trichomoniasis*

Patient no.	Age	Days in menstrual cycle	Coincidental *N gonorrh.* infection	Surface epithelium: Stratified squamous epithelium	Surface epithelium: Columnar epithelium (erosio)	Clusters of *T vag* on epithelium
449	19	90	+	+	0	0
450	18	–	0	0	+	0
451	26	§	+	+	0	0
452	20	–	0	0	+	+
497	18	14	0	+	0	+
503	20	4	0	+	0	0
523	21	10	0	+	0	+
548	21	6	+	+	0	0
551	19	7	0	+	0	0
554	23		+	0	+	0
555	23	–	0	+	0	

+ = yes 0 = no – = no information available § = pregnant, menstruation 8 months ago

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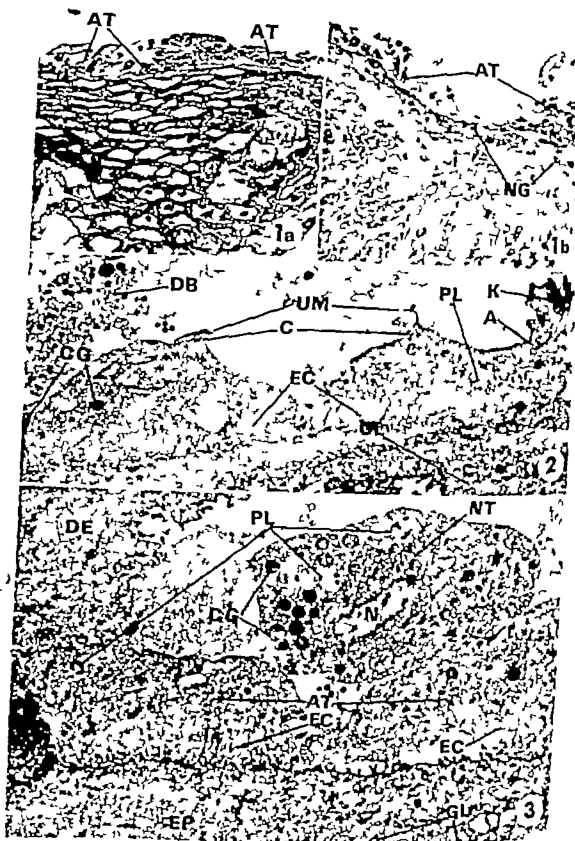
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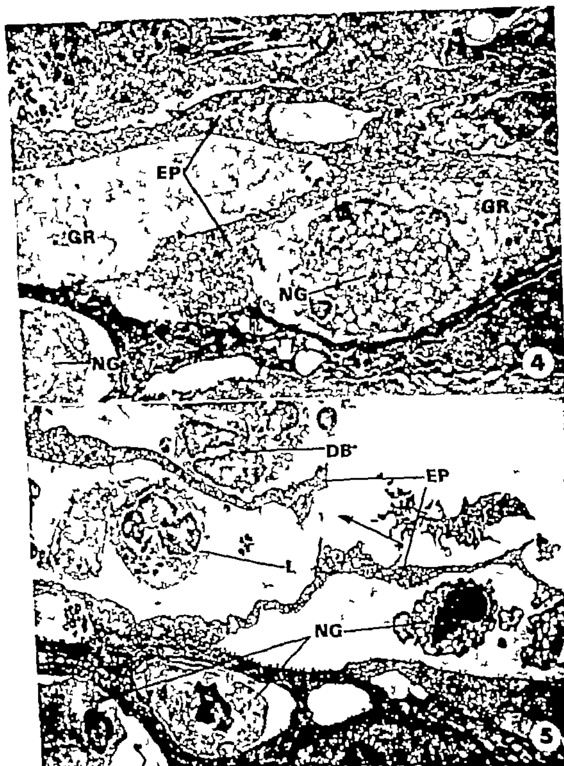
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AT
AT
AT
NG
1a
1b
DB
UM
PL
K
A
C
CG
EC
2
DE
PL
NT
N
AT
EC
EC
EP
GL
3



EP
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L
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herent cover on the vaginal surface often separated by a gap which was approximately 2 nm wide (GP Fig 7 8)

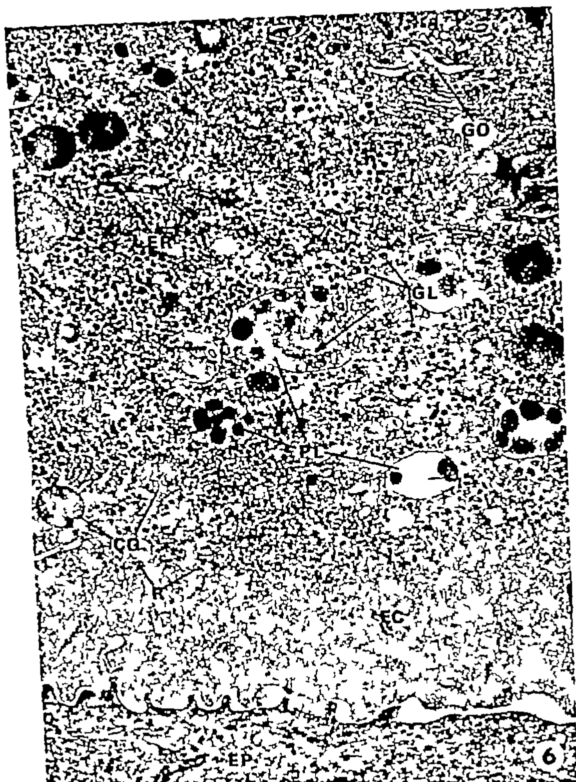
Amoeboid cells of *T vag* attached to the epithelium were found to have cell contact with necrotic epithelial cells only (Figs. 6 7 8 9 10) They were often attached to the under surface of partly desquamated surface epithelial cells but they never penetrated into intact epithelium. Beneath large cell clusters, trichomonads from patients Nos. 450 and 497 had cytoplasmic extensions into single epithelial cells (arrows, Fig 12)

Amoeboid trichomonads which were attached to the epithelium were flat cells, sometimes planoconvex, with the undulating membrane and flagella projecting from the convex cell surface (Fig 2)

Free trichomonads in the vaginal fluid and amoeboid cells attached to the epithelium contained cytoplasmic organelles of the same types as those commonly found in axenic *T vag* cultures (33) However the amoeboid cells showed a characteristic intracellular organization since a large part of their cytoplasm, viz. in an average a 1 4 μm wide zone adjacent to the epithelium (EC, Figs. 2, 3 6, 7) showed almost none of the ordinary cytoplasmic organelles but a dense network of microfilaments (MF Figs. 9 10) At the nuclear level, the network of microfilaments occupied approximately 25 per cent of the area of cross sectioned trichomonads. The microfilaments had diameters of approximately 2–5 nm. They were mostly randomly oriented but well-defined bundles located almost at right angles to the surface membrane were seen in many cells (arrows, Fig 10) particularly in the cytoplasm opposite to remnants of desmosomal attachment plaques or tonofilaments in adjacent epithelial cells. The trichomonad surface membrane was closely and broadly attached to the underlying epithelium and often interdigitated with the small projections of the epithelial cell membrane (Figs. 6, 9 10) The space between the surface membranes of the trichomonads and the epithelium was occasionally partly obliterated (arrow Fig 9 JU Fig 10)

The *mastigont* of trichomonads in the biopsies was usually identical with that of cultivated cells (33) (Fig 13) Duplicated axostyles were seen more frequently and intracytoplasmic flagellar *axonemes* a few times. Protrusion of the axostyle at the posterior end was not seen. The *nucleus* measured approximately 3.4 μm by 1.6 μm, and usually contained from two to five nucleoli. The *Golgi apparatus* was located near the convex cell surface of amoeboid trichomonads. In a few cells the central parallel Golgi cisterns were separated by a 9 nm wide layer of electron-dense material. Some of the Golgi cisterns and many vesicles had a content of low electron density while a few contained some very dense material. The *chromatic granules* showed no distinct paracostal or paraxostylar layering They were spherical (CG Figs. 2, 3) and showed a coarse granular matrix (CG Fig 10) except for a small part at the periphery where they were more intensely stained and presented a homogeneous appearance (D Fig. 10) *The granular endoplasmic reticulum* was located in the perinuclear cytoplasm. It had a content of low electron density *Smooth endoplasmic reticulum* in the form of tubular cisterns and/or small vesicles was often confined to the Golgi region. The number of *free ribosomes* far exceeded the number of ribosomes which were attached to the endoplasmic reticulum. They were the only granular structure which could be present in the cytoplasm otherwise occupied by microfilaments. *Phagosomes* were recognized due to their content of ingested material (PL, Figs. 2. 3 6) Most of this was mycoplasms (M Fig 15) and sometimes cell debris from trichomonad cells. *More rarely*

Fig 6 Part of amoeboid *T vag* cell and epithelial cell (EP) The homogeneously stained ectoplasm of the trichomonad cell (EC) is sharply demarcated from the granular cytoplasm, which contains clusters of ribosomes (R) granular endoplasmic reticulum (ER) chromatic granules (CG) phagosomes with mycoplasms (PL) glycogen granules (GL) and Golgi apparatus (GO) A lysed mycoplasm is marked with an arrow Magn. 28,000 ×


GO
ER
GL
PL
CG
R
EC
EP
6

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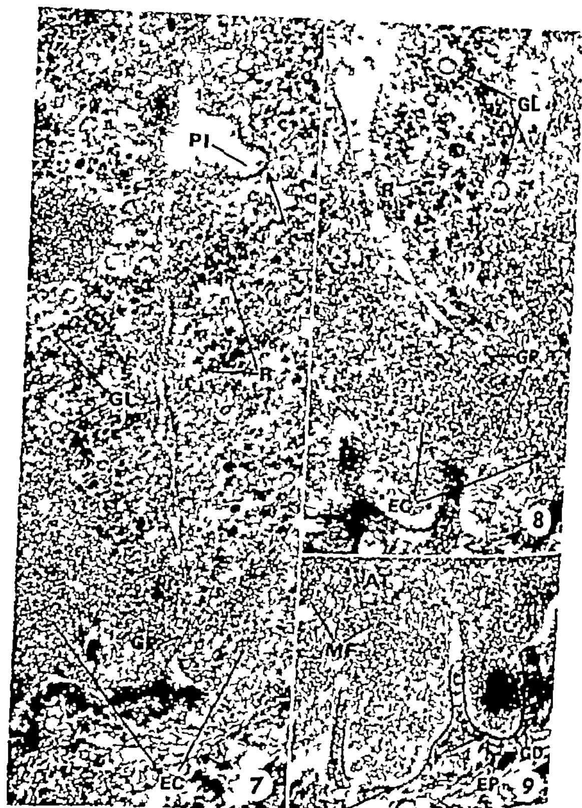
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ER
GL
PL
CG
R
EC
EP


PI
GL
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R
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8
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MF
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EP
9

bacteria were found in the phagosomes. Cell debris of probable mycoplasmic origin was also seen (arrow Fig 6)

Pinocytotic invaginations and vesicles were numerous in amoeboid trichomonads. They were found all over the cell surface, except for the part which was attached to the epithelium. The limiting membrane of the pinocytotic channels or vesicles was often covered on the luminal side by a 44 nm wide cell coat (CC, Fig. 10 PI, Fig 11) The same type of cell coat was found in spots on the outside of the cell membrane (CC, Fig 11) It had a filamentous substructure.

Glycogen granules were always present in the trichomonad cells. They were located randomly in the cytoplasm (GL, Figs. 6 7 8 10, 11 13) However the "ectoplasm" of trichomonads which were attached to the epithelium contained no glycogen.

Most granules had an irregular somewhat spherical shape, the larger ones often with a cracked appearance (GL, Fig 7 8) Their size varied from 25 nm to 100 nm. The large granules were unevenly stained, with increased density of the peripheral part. Rosettes were rarely noted. The cytoplasmic matrix close to the glycogen granules was always unstained and was devoid of cytoplasmic organelles.

DISCUSSION

The present electron microscopic investigation of vaginal biopsies from patients with trichomoniasis has demonstrated cell contact between cells of *T vag* and the superficial layer of the vaginal epithelium. The trichomonads were gathered in small cell clusters on the stratified squamous epithelium but covered only a small area of the surface.

The present study of biopsy specimens has failed to demonstrate that trichomonad cells can penetrate into deeper cell layers of the stratified squamous epithelium. Other workers who have investigated the natural infection have occasionally noted invasive growth of the trichomonads, but presumably only in epithelium which was primarily affected by definite necrobiosis (24 25) Thus cells of *T vag* normally differ from cells of *Trichomonas gallinae* (43) and *Trichomonas gallinarum* (29) being unable to invade normal

Figs. 7 and 8 The minimum space between adjacent *T vag.* cells is the size of gap junctions (GP) Glycogen granules surrounded by translucent halo are marked GL. Polyribosomes are marked R The main part of the cytoplasmic microfilaments is confined to the ectoplasm (EC) facing the epithelium. PI (Fig. 7) denotes pinocytotic invagination of the coated vesicle type (arrow) Magn Fig. 7: 56 700 × Fig. 8 70,000 ×

Fig 9 Contact between *T g* cell (AT) and an epithelial cell (EP) The cell membrane of the epithelial cell is barely ible as t is closely tached to the trichomonad ell membrane (rrows and partly hidden by the marked thickening (CD) of the cell periphery in the region of the inner leaflet of the membrane MF denotes microfilaments in the trichomonad ll. Magn 61,500 ×

Fig 10 Adjacent *T vag* (AT) and epithelial cell (EP) The network of microfilaments (MF) shows regions with more well-defined bundles (arrows) One of these terminates at the cell membrane. The surface membranes of *T vag* and the epithelial cell are in direct contact (JU) Part of the chromatic granule (CG) has an amorphous appearance and increased lectron density (D) Part of the pinocytotic invagination (PI) is covered by a cell-coat (CO) Glycogen granules (GL) are surrounded by an electron translucent zone. Magn. 62,000 ×

Fig 11 The cell coat (CC) is visible on the cell surface of two trichomonad cells and in a pinocytotic invagination/vesicle (PI) The cross-cut anterior flagella with nine pairs of microtubules and two single microtubules are marked AF GL denotes glycogen. Magn. 54,000 ×

Fig 12 Trichomonad cell (AT) invading epithelial cell (EP) Extensions of trichomonad ectoplasm are marked by arrows. GL denotes glycogen granules in the epithelial cell and T tonofilaments. Magn. 70,000 ×

Fig 13 Mastigont organelles of amoeboid *T v g* cell: axostyle (A) basal bodies/flagella (K) and pelta (P) Ribosomes confined to the concave face of the axostyle are marked (R) GL denotes glycogen granules. Magn. 40,000 ×


GL
CG
D
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GL
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11


GL
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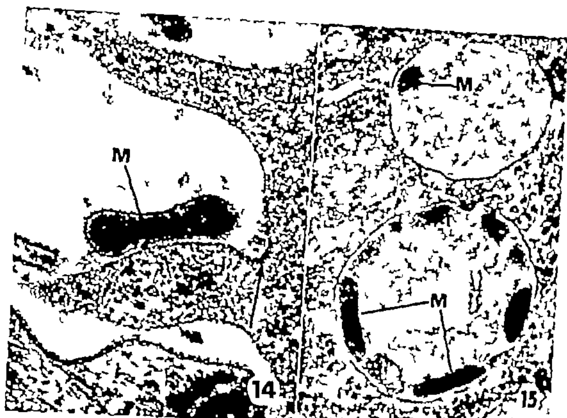


Fig 14 Mycoplasm (M) attached to the invaginating cell surface of a *T vag* cell (arrow) Magn. 56,000 ×

Fig 15 Phagosomes with mycoplasms (M) attached to the limiting membrane Magn. 32,000

tissue of the natural host. In experimentally infected animals, however pathogenic strains of *T vag* invade the tissue, with consequent inflammation and destruction of the host cells (7 15) a situation which histologically is difficult to distinguish from the "necrobiosis" occasionally observed in human vaginal epithelium.

Cells of *T vag* were often found inside non-phagocytizing cells of cell cultures (12) of experimentally infected mice (7 14) and occasionally of the vaginal epithelium (14) We have observed cells of *T vag* which were invading squamous epithelial cells, but as only the superficial layer of the epithelium and the partly desquamated cells were attacked we are inclined to believe that the strains of *T vag* examined were able to attack only necrotic cells. This was also observed in cell cultures "infected" with strains of *T vag* of moderate and low pathogenicity whereas strains with high pathogenicity presumably penetrate normal nonphagocytic cells (12)

In tissue cultures, the pathogenic effect of *T vag* cells, but not of those of *Trichomonas gallinae* (23) or *Trichomonas foetus* (28) was dependent on the attachment of the trichomonad cells to the surface of the target cells. This has been observed in many laboratories (8, 12 20 27) and therefore a "mechanical" cytotoxic mechanism has been proposed (8) It was also found that pathogenic strains of *T vag* *T gallinae* and *T foetus* affected tissue cultures by means of substances produced and liberated into the culture medium. The nature of these substances is unknown. Apparently this mode of cell interaction plays a minor role in the infection of cell cultures by *T vag* (8, 12) The present investigation has shown that the amoeboid trichomonas cell undoubtedly damages

the vaginal epithelium since epithelial lesions were located mostly beneath large cell clusters of trichomonads where a coherent layer of these cells was closely attached to the epithelium. However the clusters of *T vag* cells covered only a small part of the mucosa, and it is therefore unlikely that this damage was essential to maintain the severe vaginitis found in our patients. The more so as the local inflammatory reaction of the mucous membrane was generally the same whether or not *T vag* cells were present on the epithelial surface. Thus, in the present study the pathological changes due to the *T vag* infections were mainly due to "toxic" substances which were acting on the epithelium independent of contact with *T vag* cells, and which presumably could just as well have been formed by the free non-amoeboid trichomonads. A similar effect of *T vag* cells was noted in experimentally infected mice, where the trichomonads damage host cells without establishing any direct contact (7)

It is well known from light microscopic studies that, in contrast to the animal pathogen *T gallinae* (23) and *T foetus* (28) cells of *T vag* are often attached to the surface of cells (8, 12, 20 27) to cell debris or other objects (18, 22) However to our knowledge, the fine structure of the amoeboid trichomonads which were attached to other cells has not been described until recently (7)

The thick, otherwise organelle free, part of the cytoplasm which is formed by the dense network of microfilaments corresponds morphologically to the "ectoplasm" or "hyaloplasm" of other migrating protozoa (6 9 39 40 48) or metazoan cells (2, 42 45) The coherent layer of microfilament which forms the ectoplasm" of the *T vag* cells was in this study found to occupy a comparatively large part of the cell. This is normally found only in the case of a few protozoa moving on solid surfaces (17) and never in cases of migratory tissue cells studied *in vitro* In the latter the microfilament reticulum is confined to the ruffles (45) or lamellipods at the moving cell edge only (42)

Cells of the non-amoeboid *T vag* have only a few visible cytoplasmic microfilaments and show no "ectoplasm" except occasionally around large phagosomes (35) In such cases they are probably induced by a surface contact mechanism, analogous with the microfilaments active in phagocytotic processes of other cells (3) A similar mechanism may be responsible for the formation of microfilaments in the amoeboid cells of *T vag*., as it could explain why the microfilaments were confined almost exclusively to the part of the trichomonad cell in direct contact with an epithelial cell.

It is not known whether the microfilaments are active in the change of cell shape which takes place in relation to cell motility However we assume that the trichomonad cell adheres closely to and applies a strong force of tension onto the underlying epithelium. In this way *T vag* cells might mechanically damage the limiting membranes of the epithelial cells and give rise to deformation of the target cells (18) or to the "mechanical cell damage" previously observed if *T vag* cells are attached to the cells of cell cultures (8, 12, 27)

Since endocytotic vesicles only were numerous in connection with the free cell surface of the *T vag* cells, we may conclude that the ectoplasm is not active in cellular endo- or exocytosis.

The particulate content of the phagosomes were almost exclusively mycoplasms. This may be because mycoplasms, in addition to the trichomonads, were the dominating microorganism in the vaginal fluid adjacent to the epithelium. Others have shown that mycoplasms often become attached to the vaginal epithelium (31) and, in the present biopsies, almost no bacteria were seen near the epithelial surface. Mycoplasma (*M hominis*) and *T vag* are considered to be pathogenic microorganisms in the female genital tract, and, as they often are present simultaneously (13 31) the role of each of these is difficult to assess. The present findings do not elucidate this problem.

Pinocytotic invaginations were formed from the free cell surface and frequently from

43 *Stabler R M* *Trichomonas gallinae* A review Exptl. Parasitol. *3* 368–402, 1954

44 *Trussel R E. & Plass D E.*. The pathogenicity and physiology of pure culture of *Trichomonas vaginalis*. Am. J Obst. Gynec. *40* 883–890, 1940

45. *Wessells N K* *Spooner B. S* *& Ludueña M A*. Surface movements, microfilaments and cell locomotion. Locomotion in Tissue Cells. Ciba Foundation Symposium 14 (New Series) Associated Scientific Publishers, Amsterdam, Holland, 1973.

46 *Vickerman, K.*. On the surface coat and flagellar adhesion in trypanosomes. J Cell Sci 5 163–193 1969

47 *Vye M V* *& Fischman, D A* A comparative study of three methods for ultrastructural demonstration of glycogen in thin sections. J Cell. Sci. *9* 727–749 1971

48. *Zaman V.*. Structure and movement of entamoeba. Ann. Trop. Med. Parasit. 66 329–333 1972.

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THE SEROLOGY OF *PSEUDOMONAS AERUGINOSA* ANALYSED BY MEANS OF QUANTITATIVE IMMUNOELECTROPHORETIC METHODS

I Comparison of Thirteen O Groups of Ps. aeruginosa *with a Polyvalent* Ps. aeruginosa *Antigen Antibody Reference System*

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Høiby N The serology of *Pseudomonas aeruginosa* analysed by means of quantitative immunoelectrophoretic methods. I Comparison of thirteen O groups of *Ps. aeruginosa* with a polyvalent *Ps. aeruginosa* antigen-antibody reference system. Acta path. microbiol scand. Sect. B, 83 321 327 1975

Serologic cross-reactions between 16 strains of *Pseudomonas aeruginosa* representing 13 O groups were studied by various quantitative immunoelectrophoretic techniques. As reference system was used a polyvalent *Ps aeruginosa* antigen and a corresponding rabbit antiserum. Fifty-one (93 per cent) of the 55 *Ps. aeruginosa* antigens in the reference system were present in all the strains and corresponding antibodies in the reference system could be completely absorbed by all the strains. Complete cross-reactivity was also found between antigens of the reference system and 3 of the 4 antigens present only in some of the strains The last of the 4 antigens not present in all the strains could only absorb part of the corresponding antibodies in the reference system. Absorption experiments with whole heat killed bacteria indicate that this antigen is related to the O group antigens of *Ps. aeruginosa*. None of the antigens of the reference system were related to the mucoid substance produced by some strains of this bacterium.

Key words: *Pseudomonas aeruginosa* serology thirteen O groups; quantitative immunoelectrophoresis.

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Pseudomonas aeruginosa (*Ps aeruginosa*) isolated from human sources have a rather complex serology as illustrated by O group schemes and analyses by means of crossed immunoelectrophoresis (5 6, 7 9 12, 13) The antigenic complexity is also evident when the antibody response in patients chronically infected with this bacterium is investigated (4 7 8) The antigenic complexity of *Ps aeruginosa* raises therefore problems as regards choice of antigen prep-

aration for investigation of the serology of *Ps aeruginosa* or the humoral immune response against this bacterium in patients suffering from *Ps aeruginosa* infection.

The present work was designed to investigate the serology of *Ps aeruginosa* by means of crossed immunoelectrophoretic methods which allow analysis of even very complex antigenic samples (1 2). By means of a previously described polyvalent *Ps aeruginosa* antigen-antibody standard reference system, 13 O groups of this species and the 4 strains of the reference-system have been compared.

MATERIALS AND METHODS

The St-Ag/St-Ab Reference System

As reference system in the immunoelectrophoretic analyses is used a polyvalent *Ps. aeruginosa* standard-antigen/standard-antibody system (St-Ag/St-Ab) as described previously (7). The St-Ag consists of water-soluble constituents obtained from 4 different O groups of mucoid *Ps. aeruginosa* by sonication (O group 3 3A 6 and 11). The St Ab is a pooled, concentrated rabbit antiserum against St-Ag (7). Sixty-one precipitates can be counted after crossed immunoelectrophoreses of St-Ag against St Ab, and 55 of these are regularly visible using the antigen-antibody ratio adapted in the present study (7). The precipitates have been enumerated, beginning with number 1 in the anodic end of the 1st dimension electrophoresis (9).

Antigens

Twelve non-mucoid *Ps. aeruginosa* strains representing O group 1 to 13 described by *Mikkelsen* (1968 & 1970) were obtained from the Department of Hospital Infections, Statens Seruminstitut. One of the strains belonged to both O group 7 and 8, each of the remaining 11 belonged to one single O group. Antigens were produced from these 12 strains by sonication according to previously described principles (7 9). Protein concentration of these antigens was 4 13 g per litre (Biuret method). Production of antigens of each of the 4 strains of St-Ag and protein concentrations has been described previously (7). Antigens were also produced from 10 *P aeruginosa* strains—mucoid and non-mucoid—recently isolated from clinical material. These strains belonged to O group 2, 5A, 6 and 10A according to *Mikkelsen*'s (1970) classification. Protein concentration were 11 18 g per litre. Six of these strains have been described elsewhere (10).

Preparation of Heat-killed Ps. aeruginosa Cells for Absorption Experiments

Each of the 12 O groups strains and the 4 strains of St-Ag (designated St-Ag 0-3 to St-Ag 0-11) were cultured on solid media (Tröche) and harvested as described previously (7) and suspended in 0.154 M sterile NaCl. One portion of each suspension was heat-killed at 100° C for 1 h and another portion heat-killed at 120° C for 2 h according to the principles used by *Mikkelsen* (1970) and *Homma* (1974) for preparation of cells for O-agglutination. After heat-killing, the cells were washed twice in sterile saline and equal volumes of bacterial pellet and St-Ab were mixed. The absorption took place at 35 C for 1 h followed by 4 C overnight. After centrifugation (48,200 × g for 30 min) the supernatant was used in the second dimension gel of crossed immunoelectrophoresis of St-Ag and the precipitate pattern compared with a control plate with unabsorbed St Ab in the second dimension gel.

Preparation of Mucoid Substance from Ps. aeruginosa

Mucoid substance was prepared from one of the mucoid strains of St-Ag (St Ag 0-11) according to the method of *Doggett et al.* (1964) but with some modifications. The bacteria were grown on solid media, harvested as described previously (7) and mixed with half as much distilled water (v/v). This suspension was then homogenized with a glass homogenizer and the cells pelleted at 48,200 × g for 1 h repeated 4 times and finally at 120,000 × g for 90 min. The resulting very viscous supernatant was dialysed against distilled water for 3 days at 4 C. The presence of mucoid substance in the viscous solution could be demonstrated by ethanol precipitation as described by *Doggett et al.* (1964). The solution contained 4 mg dry weight material per ml, and agarose gel electrophoresis stained for proteins (7) revealed no visible protein bands. Mucoid substance was used in absorption experiments similar to those described above and for immunoelectrophoretic studies described below.

Immunoelectrophoretic Methods

Antigens from each of the *Ps aeruginosa* strains and mucoid substance were run 1) in crossed immunoelectrophoresis against St-Ab (8) and 2) in crossed-line immunoelectrophoresis the antigen in question being included in an intermediate gel between 1 and 2 dimension electrophoresis of St-Ag against St-Ab (absorption of antibodies in situ) (1 2 9 11). These electrophoreses were repeated at least twice with each of the antigen preparations, and as controls crossed-line immunoelectrophoresis was run with St-Ag or saline included in the intermediate gel. The percentage of antibodies absorbed in the crossed-line immunoelectrophoresis

(absorption of antibodies *in situ*) was estimated by comparison with set of 4 standard plates containing 100% 75%-50% and 25% of the original concentration of St Ab in the second dimension gels. The increase in enclosed area by a given precipitate after absorption *in situ* was then expressed as 0% 25%≤–<50% 50%≤–<75% 75%≤–<100% and 100% absorption of antibodies against the antigen in question if the area enclosed by a given precipitate after absorption of antibodies *in situ* was similar to the area of the same precipitate on the standard plate containing 25% of the original concentration of St Ab then 75% of the antibodies against the antigen in question had been absorbed. In this way a measure was obtained of the degree of partial identity (1 2 9) Absorption less than 25% was considered insignificant and not counted. The electrophoreses were run in microtechnique on 5 × 5 cm glass plates, using equipment and agarose as described previously (8) The first dimension electrophoreses were run with 2 µl of the antigens. The intermediate gels of the second dimension electrophoreses contained 40 µl antigen per cm (crossed-line immunoelectrophoresis) or 0 154 M N Cl. Temperature of

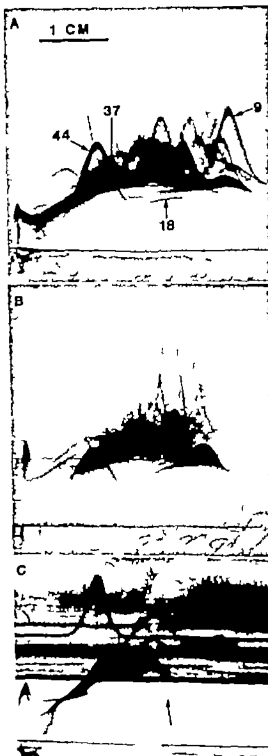


Fig. 1 Comparison of antigens from a strain of *Ps. aeruginosa* (St-Ag 0–6) using the polyvalent *Ps. aeruginosa* antigen-antibody reference system (St-Ag/St-Ab)

A. Crossed immunoelectrophoresis with intermediate gel of St-Ag against St-Ab. No antigens or antibodies were included in the intermediate gel. Precipitates corresponding to 4 "variable antigens (Table 1 & 2) are indicated by arrows and numbers.

B. Crossed immunoelectrophoresis with intermediat gel of antigens from a strain of *Ps. aeruginosa* (St-Ag 0–6) against St Ab. No antibodies or antigens were included in the intermediate gel. Precipitates resembling numbers 9 18 and 37 cannot be seen in the pattern when comparing with *A*

C Crossed-line immunoelectrophoresis of St-Ag in the well and antigens from the strain St-Ag 0–6 in the intermediate gel against St Ab in the reference gel (absorption of antibodies *in situ*) Most of the precipitates have been 'elevated' or removed from the reference pattern. Straight precipitate lines showing reaction of identity with the elevated reference precipitates are seen in the gel. Three of the reference precipitates indicated by arrows (numbers 9 18 and 37) has not been influenced by the antigens from the intermediate gel (compare *A* and *C*) thus confirming the absence of these 3 antigens in the strain St-Ag 0–6. (Technical 1 dimension electrophoresis anode to the right. 2 dimension lectrophoresis anode at the top Staining Coomassie brilliant blue)

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TABLE 1 *Occurrence of 55 Antigens in 12 Strains of Ps. aeruginosa Representing 13 O Groups and in the 4 Strains of Pseudomonas aeruginosa Used in a Polyvalent Antigen (St Ag) and Corresponding Antibody (St Ab)*

Antigen number (No.) and percentage of cross-reactivity in relation to the reference St-Ag/St Ab pattern

O group	No. 9 (%)	No. 18 (%)	No. 37 (%)	No. 44 (%)	Remaining 51 antigens (%)
1	0	0	50–75	100	100
2	100	0	0	100	100
3	100	0	25–50	100	100
4	100	0	0	100	100
5A	100	0	0	100	100
6	0	0	0	100	100
7/8	100	0	0	0/100	100
9	0	0	0	100	100
10A	100	0	0	100	100
11	100	0	0	100	100
12	0	0	0	100	100
13	100	0	0	0	100
St-Ag 0–3	0	0	50–75	100	100
St-Ag 0–5A	100	100	50–75	100	100
St-Ag 0–6	0	0	0	100	100
St-Ag 0–11	100	100	0	0	100
St-Ag (control)	100	100	100	100	100

agarose when antigen was added: 40 C. Washing drying and staining of gels as reported previously (7 8)

RESULTS

Table 1 shows the results of the immunoelectrophoretic analyses of the 12 strains representing the 13 O groups and the 4 strains of St Ag An example of an analytic series of immunoelectrophoreses is seen in Fig 1 It is seen that 51 (93 per cent) of the antigens are found in all the strains investigated regardless of O group, whereas 4 (7 per cent) of the antigens were found only in some of the strains. Two of these antigens (numbers 18 and 37) were found only in a few of the O groups, and antigen number 18 was found solely in mucoid strains. Any relationship between the concentration of the antigen samples and the presence or absence of the 4 variable antigens was not found. All the strains had at least 52 (95 per cent) of the antigens, but none possessed all the 55 antigens, the closest was St Ag 0–5A which could absorb all antibodies against St-Ag except 25–50 per cent of the antibodies against antigen number 37

Two batches of antigens were produced from each of the 12 O group strains given in Table 1 Results obtained by each of the batches were identical except with respect to the strain representing O group 7/8. The second batch from this strain contained antigen number 44 which was absent in the first batch. A third batch of antigens from this strain was produced, and this batch also contained antigen number 44 All the immunoelectrophoreses were repeated at least twice with identical results

The experiments with the 10 *Ps aeruginosa* strains recently isolated from clinical material showed similar results (Table 2) All strains had at least 52 of the antigens in St Ag and only antigen number 9 18, and 37 was sometimes absent and antigen number 18 was found only in one of the mucoid strains.

TABLE 2. *Occurrence of 35 Antigens in 10 Strains of Pseudomonas aeruginosa Recently Isolated from Clinical Materials*

		Antigen number (No.) and percentage of cross-reactivity in relation to the reference St-Ag/St-Ab pattern			
O group	Mucoid/ Non-mucoid	No. 9 (%)	No. 18 (%)	No. 37 (%)	Remaining 32 antigens (%)
2/5A	Non-mucoid	100	0	0	100
5A	Non-mucoid	100	0	0	100
6	Non-mucoid	100	0	0	100
6	Mucoid	100	100	0	100
6*	Mucoid	100	0	0	100
6/10A	Mucoid	100	0	0	100
10A	Non-mucoid	0	0	0	100
10A	Mucoid	100	0	0	100
10A	Mucoid	100	0	50–75	100
10A	Non-mucoid	0	0	0	100

These strains are further described elsewhere (10)

The absorption experiments with heat killed whole bacterial cells showed that these cells could solely absorb *antibodies against antigen number 37 (a broad, characteristic* precipitate in the reference system (Fig 1 A)) The results of these absorption experiments were in accordance with the results of the absorption *in situ experiments* (Table 1) as regards antigen number 37 *i.e.* only cells from O group 1 and 3 and of St-Ag 0–3 and St Ag 0–5A could absorp antibodies to antigen number 37 in St-Ab. Any differences between absorption *experiments with* 100 C and 120 C heat killed cells was not found. Results obtained by heat killed mucoid and non-mucoid variants of St Ag 0–11 were identical.

Mucoid substance was *not able to absorb* antibodies in St-Ab *against any of the antigens* of St Ag, and mucoid substance did not precipitate with St Ab in crossed immunoelectrophoresis or crossed-line immunoelectrophoresis.

DISCUSSION

The results show that the previously described antigenic complexity of the polyvalent St Ag (7) is caused by a similar complexity of each of the *Ps. aeruginosa* strains investigated, and the majority of the antigens were common to all the strains regardless of O group. Further more, the qualitative antigenic composition of each strain of *Ps. aeruginosa* seems to be stable and reproducible, as the results obtained *by different batches of the same strain* showed.

Three of the 4 variable antigens (number 9 18, and 44) were either present or absent as *judged by* absorption of *antibodies in in situ* experiments, whereas the situation was different as regards antigen number 37 This antigen was found only in 5 of the strains (Table 1 & 2) and each of these could only absorb part of the antibodies in St-Ab corresponding to antigen number 37 This means that none of the strains possessed all the antigenic determinants of antigen number 37 present in St Ag which is a mixture of 4 strains. Moreover the absorption experiments with whole bacterial cells, heat killed as for O-agglutination showed that antigen *number* 37 is situated on the *surface of the cells and is* thermostable. Other experiments (unpublished results) have shown that antigen number 37 is the only antigen in St Ag which resists 120 C for 2 h and 100 C *for* 1 h leaves only traces of other antigens in St-Ag. It is therefore likely that antigen number 37 *in the reference system is* related to some of the antigenic determinants used in the O group typing *of Ps aeruginosa*.

In this connection it must be stressed that

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7/8	100	0	0	0/100	100
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10A	100	0	0	100	100
11	100	0	0	100	100
12	0	0	0	100	100
13	100	0	0	0	100
St Ag 0–3	0	0	50–75	100	100
St Ag 0–5A	100	100	50–75	100	100
St Ag 0–6	0	0	0	100	100
St Ag 0–11	100	100	0	0	100
St-Ag (control)	100	100	100	100	100

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The experiments with the 10 *Ps. aeruginosa* strains recently isolated from clinical material showed similar results (Table 2) All strains had at least 52 of the antigens in St Ag and only antigen number 9 18 and 37 was sometimes absent, and antigen number 18 was found only in one of the mucoid strains.

In Axelsen, N. H., Krøll, J. & Weeke, B. (Eds.) *A Manual of Quantitative Immunoelectrophoresis.* Universitetsforlaget, Oslo 1973 p. 79–81

12. *Mikkelsen, O. S.* Serotyping of *Pseudomonas aeruginosa.* 1. Studies on the production of anti O sera. Acta path. microbiol. scand. *73* 373–390, 1968.

13. *Mikkelsen, O. S.:* Serotyping of *Pseudomonas aeruginosa.* 2. Results of an O group classification. Acta path. microbiol. scand. Sect. B, *78* 163–173 1970.

Acta path. microbiol. scand. Sect. B, 83: 328–334, 1975

THE SEROLOGY OF *PSEUDOMONAS AERUGINOSA* ANALYSED BY MEANS OF QUANTITATIVE IMMUNOELECTROPHORETIC METHODS

II. Comparison of the Antibody Response in Man Against Thirteen O Groups of Ps. aeruginosa

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Høiby, N. The serology of *Pseudomonas aeruginosa* analysed by means of quantitative immunoelectrophoretic methods. II. Comparison of the antibody response in man against thirteen O groups of *Ps. aeruginosa*. Acta path. microbiol. scand. Sect. B, 83: 328–334, 1975.

The occurrence of antibodies against antigens prepared from strains representing 13 O groups of *Pseudomonas aeruginosa* and against a polyvalent *Ps. aeruginosa* antigen (St-Ag) has been investigated in sera from 100 patients. By means of fused rocket immunoelectrophoresis with intermediate gel it was found that the humoral immune response against *Ps. aeruginosa* resulting in precipitating antibodies will be detected by St-Ag as well as by any other of the antigen samples investigated. Six of the sera contained group-specific antibodies which were revealed by only one of the antigen samples used and not by St-Ag. These six sera were further studied by means of various quantitative immunoelectrophoretic methods using St-Ag as well as antigens prepared from the infecting *Ps. aeruginosa* strain in the patient concerned. In all six sera, only one extra precipitin could be detected using antigens prepared from the homologous strain instead of St-Ag. This extra precipitin corresponded presumably to group-specific O-antigens not included in St-Ag. In sera from patients, these group-specific antibodies were always accompanied by antibodies against antigens common to all strains of *P. aeruginosa*.

Key words: *Pseudomonas aeruginosa*; serology; quantitative immunoelectrophoretic methods.

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The complex antigenic structure of *Pseudomonas aeruginosa* (*Ps. aeruginosa*) raises problems as regards choice of antigen preparation in studies of the patients' (pts.) immune response against this bacterium (6, 7, 13, 14). To overcome these problems, a polyvalent standard antigen (St-Ag) has been used in previous works (7, 8). St-Ag consists of the four O groups of *Ps. aeruginosa* most frequently isolated from the patients included in these studies (7). However, at least the group-specific antigens of other O groups are not included in St-Ag.

In a previous article (11) it has been shown that 93 per cent of the antigens in St-Ag are found in all the strains investigated, which represented 13 of the O groups described by *Mikkelsen* (1970) but none of the strains possessed all the antigens of St-Ag The conclusions of that study however were limited to the antigens present in St Ag (11)

The present study was undertaken to investigate whether *Ps. aeruginosa* antigens exist— besides O antigens— which are not present in St Ag Such antigens might induce precipitating antibodies which will escape detection with St Ag. To investigate this possibility sera from 100 patients have been examined by means of various immunoelectrophoretic methods for precipitating antibodies against St-Ag and against antigens made from *Ps ae ruginosa* strains representing 13 of the O groups described by *Mikkelsen* (1970)

MATERIALS AND METHODS

The St-Ag/St-Ab Reference System

As reference system in the immunoelectrophoretic analyses, a polyvalent *Ps aeruginosa* standard-antigen /standard-antibody system (St-Ag/St-Ab) is used. St-Ag is obtained by sonication of four O groups (3 5A, 6, and 11) of mucoid strains, and St-Ab is a pooled, concentrated rabbit antiserum against St-Ag. This reference system has been described elsewhere (7)

Antigens Representing 13 O G oups of Ps. aeruginosa

Antigens were prepared from 12 non-mucoid *Ps. aeruginosa* strains representing O group 1–13 in *Mikk lsen* (1970) system. One of the strains belonged to both O groups 7 and 8, each of the remaining 11 belonged to one single O group. Antigens were also prepared from 6 *Ps. aeruginosa* strains isolated from six of the patients included in the present study Antigens were produced from these 18 strains by sonication as described elsewhere (11)

Patient and Ser

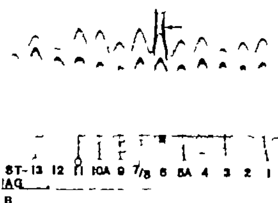
Sera were obtained from 100 patients 65 of these suffered from cystic fibrosis (CF) and 35 suffered from other diseases (non-CF) Fifty-one of the CF patients were harbouring or had previously harboured *Ps aeruginosa* in the respiratory tract at he time of blood-sampling Fourteen of the CF patients had, to our knowledge never harboured these bacteria in the respiratory tract, but these patients had suffered from recurrent respiratory tract infections caused by other bacteria (9) The 35 non-CF patients had all been infected or colonized by *Ps. aeruginosa* in the respiratory tract or elsewhere. These 100 sera were selected from a large collection of sera which had been examined for precipitating antibodies against St-Ag by means of crossed immunoelectrophoresis (8) Fifty of the sera contained precipitating antibodies against St-Ag and 50 contained no detectable antibodies against St-Ag In The total number of antibodies against St-Ag in the 50 'positi sera was 481 (mean 9 range 1–40) Serotyping of the *Ps. aeruginosa* strains isolated from the 86 patients who had harboured this species was kindly performed by the Department of Hospital Infections, Statens Seruminstitut. All isolates were stored as described by *Mikkelsen* (1970) All O groups except 7 8, 12, 13, and 14 were represented among these isolates. Sera were stored at −30° C with NaN_3 added to a concentration of 15 mM.

Immunoelectrophoretic Methods

One µl of each of the 12 antigenic samples representing the 13 O groups and 1 µl St-Ag were run in *fused rocket immunoelectrophoresis with intermediate gel* (1 2 15) against an intermediate gel containing St-Ab (15 µl per cm²) or 0.154 M NaCl as control and a reference gel containing serum from patients (5 µl per cm) The distance between the antigen containing wells was 5 mm and the plates were left for diffusion of the antigens at 4 C for 120 min in humid chamber prior to electrophoresis. The electrophoreses were run for 20 h t 2 V per cm, on 10 × 10 cm glass plates using equipment and agarose as described previously Thickness of gel 1.5 mm. Washing, drying and staining of gels as reported previously (7)

The St-Ag/St-Ab system was compared with antigens from six *Ps. aeruginosa* strains isolated from patients included in this study and sera from the same patient by means of comparative series of *crossed immunoelectrophoreses* and *crossed-line immunoelect phoreses* (1 3 4 8, 10, 12) in agarose gel on 5 × 5 cm glass plates An example of such series of immunoelectrophoreses is given in Figs 2 & 3 One µl of St-Ag or antigen from the strain in the patients was run against a second dimension intermediate gel containing antigen from the strain in patients (?0 µl per cm) or St Ag (20 µl per cm²) or 0 154 M NaCl as control. The second dimension reference gel contained St-Ab (10 µl per cm²) or serum from patients (10 µl per cm²) This series of immunoelectrophoreses allow comparison of patients antibodies against the infecting strain and against St-Ag, nd will reveal whether any of the patients antibodies are specific to the strain in the patient concerned (Fig 3 A-B-C) Moreover

A 1 CM



B

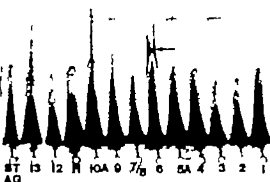


Fig 1 Fused rocket immunoelectrophoresis with intermediate gel of 12 antigen samples representing 13 O groups of *Ps. aeruginosa* and St-Ag in the 13 wells (indicated by numbers) The intermediate gels contain saline (*A*) or St-Ab (*B*) The reference gels contain serum from patients.
A Precipitates are seen corresponding to each of the 13 antigen samples. All the precipitates except one (indicated by an arrow) show reactions of identity with 'neighbour' precipitates.
B St-Ab in the intermediate gel have precipitated with the antigens in the 13 antigen samples leaving only the extra antigen in O group 6 (*A*) for precipitation with antibodies in serum from the patients (arrow)
(Technical Anode at the top. Staining Coomassie brilliant blue. Due to electroendosmosis and the broad intermediate gel, the precipitates between antibodies in the reference gels and the antigens are found in the anodic part of the intermediate gels)

this series allow comparison of patients strain with the St Ag/St Ab reference system (Fig. 2 A B-C) The technical details and chemical concerning the immunoelectrophoreses have been reported previously (8, 10)

RESULTS

The results of the 100 pairs of fused rocket immunoelectrophoreses with intermediate gels containing either saline or St Ab showed a high degree of accordance between patients antibodies detected by St Ag and by antigens from each of the strains representing the 13 O groups. Within all the 50 sera known to contain antibodies against St-Ag the fused rocket immunoelectrophoreses with saline in the intermediate gels showed the presence of antibodies against St Ag as well as against the 12 other antigen samples used. Reactions of identity between precipitates with all the 12 antigen samples were seen, and the patterns of precipitates were similar from one antigen sample to another (Fig 1) In the 50 sera which were negative with St-Ag it was not possible to detect any antibodies with any of the other antigens used.

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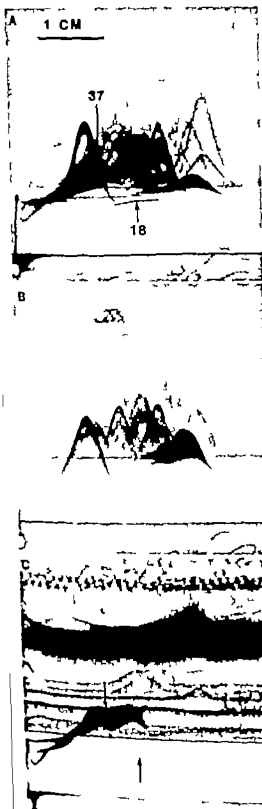


Fig 2 Comparison of antigens from a strain of *Ps. aeruginosa* (O group 6/10A isolated from a patient) with the polyvalent *Ps. aeruginosa* antigen antibody reference system (St-Ag/St Ab)

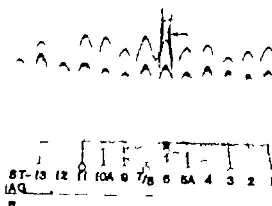
A Crossed immunoelectrophoresis with intermediate gel of St-Ag against St-Ab. No antigens or antibodies were included in the intermediate gel. Two precipitates are indicated by arrows and numbers.

B Crossed immunoelectrophoresis with intermediate gel of antigens from a patient's *Ps. aeruginosa* strain against St-Ab. No antibodies or antigens were included in the intermediate gel. Precipitates resembling numbers 18 and 37 cannot be seen in the pattern when comparing with *A*

C: Crossed-line immunoelectrophoresis of St-Ag in the well and antigens from the patient's strain in the intermediate gel against St-Ab in the reference gel (absorption of antibodies *in situ*) Most of the precipitates have been 'elevated' or removed from the reference pattern. Straight precipitate lines showing reaction of identity with the elevated reference precipitates are seen in the gel. Two of the reference precipitates indicated by arrows (number 18 and 37) have not been influenced by the antigens from the intermediate gel (compare *A* and *C*) thus confirming the absence of these 2 antigens in the strain O group 6/10A. (Technical: 1 dimension electrophoresis: anode to the right. 2. dimension electrophoresis anode at the top. Staining as Fig. 1)

sera) and 9 Corresponding isolated strains from the patients belonged to O group 5A, 6, 6, 6/10A, 10A and 6.

Antigens were produced from the strains isolated from these six patients and six comparative series of immunoelectrophoreses were run in the same way as the series shown in Fig 2 & 3 In this way it was demonstrated that each of the six sera contained only one extra precipitin which was detected by antigens from the patient's own strain and not by St-Ag, and all these six sera contained additional common *Ps. aeruginosa* precipitins which were detected by St Ag. Thus, the six sera contained 51 precipitins against *Ps. aeruginosa* and 45 (88 per cent) of these precipitins were detected by St Ag. In all cases, the 'extra precipitin detected by the patient's own strain corresponded in location and appearance to the characteristic precipitate number 37 in the reference system. Sera



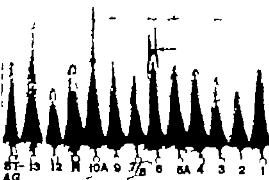


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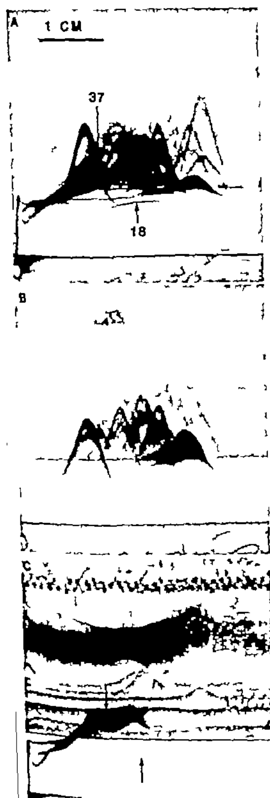


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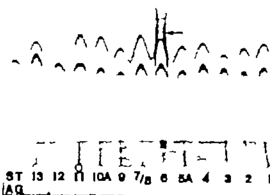
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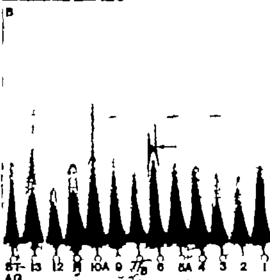


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each one of the O groups investigated. The reason why is that the vast majority of these precipitating antibodies correspond to common *Ps. aeruginosa* antigens (11)

A few precipitating antibodies are, however not detected using St Ag or any of the antigen samples produced from any single *Ps. aeruginosa* strain. These extra antibodies precipitate with antigens from the patients own strains and these precipitates resemble precipitate number 37 in the reference system in all the investigated cases. Antigen number 37 seems to be rather group-specific as indicated by the results of this study and a previous study (11) Antigen number 37 which seems to be representative for these group-specific antigens is moreover heat-stable and is probably related to some of the heat stable O antigens used for serotyping of this species (11 13 14)

Sera from patients harbouring *Ps aeruginosa* of O groups 7 8 12 13 and 14 were not available for the present study but considering the results obtained in this study and in a previous study (11) it seems reasonable to assume that the only 'extra' precipitins to be revealed by the homologous strain would be O group specific antibodies similar to those of precipitate number 37 in the reference system.

The conclusion of the present study is therefore that the humoral immune response against *Ps. aeruginosa* in man, as reflected by production of precipitating antibodies, will in all cases be detected by St-Ag It will probably also be detected by antigens produced by any single strain of *Ps aeruginosa* as the humoral immune response against this species always seems to include antibodies directed towards common antigens present in all strains of *Ps. aeruginosa*. In order to reveal precipitins against O group specific antigens similar to antigen number 37 in the reference system, it seems necessary to use the patient's own strain as antigen, or to use antigens from many different sero-groups as in the present work in the fused rocket immunoelectrophoreses The additional information obtained using one of these 2 approaches is quantitatively very small (1.2 per cent) but a possible significance of such information as regards resistance to infection can certainly not be ruled out (5)

This work was supported by grants from the *Thorvald Madsen Legat* the *National Danish Association Against Cystic Fibrosis* and the *Danish Medical Research Council*. Mrs. *Annie Bathsen* is thanked for skilful technical assistance.

REFERENCES

1 *Axelsen N H.*. Intermediate gel in crossed and in fused rocket immunoelectrophoresis. *In* Axelsen, *N H., Krøll, J & Weeke, B.* (Eds.) *A Manual of Quantitative Immunoelectrophoresis* Universitetsforlaget, Oslo 1973, p. 71–77

2 *Axelsen N H Bock E. & Krøll J.*. Comparison of antisera. *In:* Axelsen, N H., Krøll, J & Weeke, B. (Eds.): *A Manual of Quantitative Immunoelectrophoresis.* Universitetsforlaget, Oslo 1973 p 101–103

3 *Bock, E. & Axelsen N H.*. The reaction of partial identity in quantitative immunoelectrophoretic patterns. J Immunol. Meth. *2* 73–87 1972.

4 *Bock E. & Axelsen, N H.*. Comparison of antigens the reaction of partial identity *In* Axelsen, N H., Krøll, J & Weeke, B. (Eds.) *A Manual of Quantitative Immunoelectrophoresis* Universitetsforlaget, Oslo 1973 p. 95–99

5. *Crowder J G Devlin H B., Fisher M & White A* Heat-stable opsonins in sera of patients with *Pseudomonas* infections. J Lab. Clin. Med. *83* 853–859 1974

6 *Doggett R G. & Harrison G. M* *Pseudomonas aeruginosa* Immune status in patients with cystic fibrosis. Infect. and Immun. *6* 628–635 1972.

7 *Høiby N H & Axelsen, N H.*: Identification and quantitation of precipitins against *Pseudomonas aeruginosa* in patients with cystic fibrosis by means of crossed immunoelectrophoresis with intermediate gel. Acta path. microbiol scand. Sect. B, *81*: 298–308, 1973.

8. *Høiby N.*. *Pseudomonas aeruginosa* infection in cystic fibrosis. Relationship between mucoid strains of *Pseudomonas aeruginosa* and the humoral immune response. Acta path. microbiol. scand. Sect. B, *82* 551–558, 1974

9 *Høiby N* Epidemiological investigations of the respiratory tract bacteriology in patients with cystic fibrosis. Acta path. microbiol. scand. Sect. B, 82: 541–550 1974

10 *Høiby N* Cross-reactions between *Pseudomo-*

nas aeruginosa and thirty-six other bacterial species. Scand. J Immunol. *94* Suppl. 2, 1975

1 *Høiby N.*: The serology of *Pseudomonas aeruginosa* analysed by means of quantitative immunoelectrophoretic methods. I Comparison of thirteen O groups of *Ps. aeruginosa* with a polyvalent *Ps. aeruginosa* antigen-antibody reference system. Acta path. microbiol. scand. Sect. B *83*: 321–327 1975

2. *Krøll J.*. Crossed-line immunoelectrophoresis. *In* Axelsen, N H., Krøll, J & Weeke, B. (Eds.) *A Manual of Quantitative Immunoelectrophoresis.* Universitetsforlaget, Oslo 1973 p. 79–81

13 *Mikkelsen, O S.*. Serotyping of *Pseudomonas aeruginosa*. 1 Studies on the production of anti O sera. Acta path. microbiol. scand. 73. 373–390 1968.

14 *Mikkelsen O S* Serotyping of *Pseudomonas aeruginosa*. 2. Results of an O group classification. Acta path. microbiol. scand. Sect. B, *78* 163–175 1970.

15 *Svendsen P J.*. Fused rocket immunoelectrophoresis. *In* Axelsen, N. H., Krøll, J & Weeke, B. (Eds.) *A Manual of Quantitative Immunoelectrophoresis* Universitetsforlaget, Oslo 1973 p. 69–70.

Acta path. microbiol. scand. Sect. B, 83 335–342, 1975

YERSINIA ENTEROCOLITICA IN SMALL RODENTS FROM NORWAY, SWEDEN AND FINLAND

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Kapperud, G *Yersinia enterocolitica* in small rodents from Norway Sweden and Finland. Acta path. microbiol. scand. Sect. B, 83 334–342, 1975

The purpose of the present investigation was to study the occurrence of *Yersinia enterocolitica* (Y.e.) among small rodents and shrews (Soricidae) from Norway Sweden and Finland. During a period of one year beginning in the autumn of 1973 animals from seven localities were examined. Twenty-four strains of Y.e. were isolated from 551 small rodents. Isolations were made from six of the seven localities and from six of the nine small rodent species examined. Pooled faeces samples from 397 animals yielded 12 strains. Faeces from 154 small rodents were separately investigated Twelve (8 per cent) of these animals harboured Y.e. No isolations were made from 52 shrews. Most of the strains showed antigenic relationship to O-serotypes previously reported from untreated drinking water in Norway Two strains were antigenically related to O-serotype 3 but differed biochemically from the strains found in man and swine. The results indicate that Y.e. has a widespread distribution among small rodents in Norway Sweden and Finland.

Key words. *Yersinia enterocolitica* small rodents; Norway Sweden Finland.

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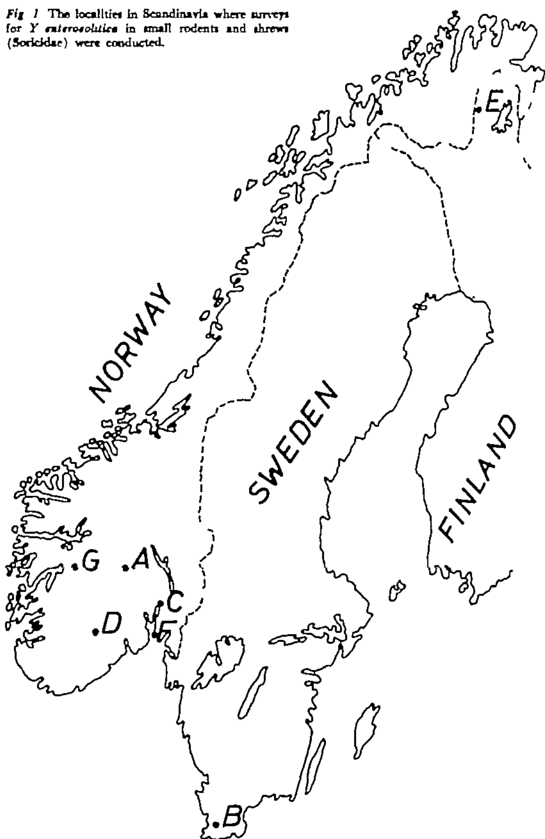
Received 5.ii.75 Accepted 5.iii.75

Yersinia enterocolitica (Y.e.) has been isolated from both man and animals, often in connection with disease, but also from apparently healthy individuals (15 16 18) Both domestic and wild animals are involved. This organism has even been found in poikilotherms (4) and has also been isolated from drinking water (12) All of this indicates that Y.e. has a wide ecological distribution.

Small mammals, especially small rodents, are important reservoirs for *Y pestis* and *Y pseudotuberculosis*. *Y enterocolitica* was recently reported to be present in small rodents from Czechoslovakia (2) The questions arise, to what extent do small rodents function as a reservoir for Y.e., and to what extent does Y.e. affect these small rodents pathologically

In the northern ecosystems small rodents display a cyclic abundance with peak populations every three to four years. Following these peak years the population density often declines rapidly Disease might be a contributing factor in the population dynamics especially during the rapid declines. On the other hand the dense and sometimes migrating populations of these animals might enable

Fig 1 The localities in Scandinavia where surveys for *Y enterocolitica* in small rodents and shrews (Soricidae) were conducted.



them to function as effective disease spreaders.

The purpose of the present investigation was to study the occurrence of Y.e. among small rodents and shrews (Soricidae) from localities in Norway, Sweden and Finland. This represents the first part of a research program with the aim of assessing small mammals as possible reservoirs for Y.e. and of Y.e. as a potential factor in the population dynamics of small rodents.

MATERIALS AND METHODS

I Collections

During a period of one year beginning in the autumn of 1973 animals from seven localities in Norway, Sweden and Finland were examined. The geographic locations are shown in Fig. 1.

Locality descriptions:

A. *Vassfaret* about 100 km north-west of Oslo, Norway. Trapping stations: Spruce, pine and spruce mixed forests. Human contacts: Many holiday cabins in the area.

B. *Stensoff* Ecological Research Station, about 20 km east of Lund, Sweden. Trapping stations: Pine plantations and groups of deciduous trees bordering open fields and pastures. Human contacts: Frequented by university personnel and students engaged in field work and field courses.

C. *Østmarka*, in the wooded area east of Oslo, Norway. Trapping stations: Mixed deciduous wood containing spruce. Human contacts: Recreational area.

D. *Kviteseid*, in Telemark, Norway. Trapping stations: Mixed forests dominated mostly by spruce. Human contacts: Many small farms and pastures in the trapping area.

E. *Kengasniemi* Northern Finland. Trapping stations: Sand banks with birch, pine and juniper bordering the river Anarjokka. Human contacts: Occasionally used for camping and fishing. Grazed by cattle.

F. *Aten* a peninsula in the Oslofjord, Norway. Trapping stations: Woods containing mostly spruce and pastures. Human contacts: Many small farms and pastures in the trapping area.

G. *Finse* on the Hardangervidda plateau, Norway. Trapping stations: Alpine tundra. Human contacts: Frequented by university personnel engaged in field work and field courses. Popular area for mountain hikers.

During the collection period most of the populations were in the pre-peak or peak years of the small rodent cycles of the northern ecosystems. Collections were made of a variety of small rodent species and shrews. Table 1 and 2 list the species and the number of individuals examined. Most animals were live trapped except for one area (Aten) where snap traps were used. Lemmings (*Lemmus lemmus*) were killed by shooting.

II Processing Collected Material

The live trapped animals were anaesthetised and blood was obtained by cardiac puncture. The heparinized blood was centrifuged and plasma was immediately frozen. From all animals faeces were subsequently extracted. The treatment of the faeces samples differed during the study. During 1973 and the beginning of 1974 faeces were pooled (Part 1). Subsequently individual faeces samples were used (Part 2).

Part 1 The primary purpose was to determine the flora of Gram negative rods with special reference to pathogens using pooled faeces samples. The present report is concerned with the results of Y.e. only. Observations on other bacterial species will be published elsewhere. Animals from six of the seven localities were examined (Table 1). Intestines were dissected out within two hours post mortem and 1–2 cm of rectum and colon were removed. Snap trapped animals were processed as soon as possible. Pools were made of up to five specimens and placed in a glycerol-saline transport medium (14). The surgical instruments were heat sterilized for each pool. Most pools had to be stored three to four days before they could be cultured.

In the following procedure all incubations were performed at 37° C for 18–22 hours unless otherwise stated: Homogenized faeces were plated onto lactose bromthymol-blue agar (Drigalski agar), desoxycholate citrate agar (without sucrose) and LSU agar (10). Enrichment was performed in selenite broth and tetrathionate broth (Kaufmann-Müller). After incubation of the broths subcultures were made on desoxycholate citrate agar and Drigalski agar. Pure culture were made from all morphologically different colonies on lactose agar. The primary biochemical characterization was carried out on the following media (14): One combined lactose-glucose-H_2S tube, one urea-indol tube and two mannitol-motility tubes. One of the mannitol-motility tubes was incubated at room temperature. The other tubes were incubated as described above. If the results indicated Y.e., the culture was selected for further serological and biochemical examination.

Part 2 The results of Part 1 showed that Y.e. was present in several samples. Consequently the interest was concentrated on this bacterial species. Animals were collected from four localities (Table 2) and faeces from each individual was cultured separately to determine the frequency of animals harbouring Y.e.

TABLE 1 Yersinia enterocolitica *in Small Mammals. Pooled Faeces Samples*

Small mammal species	Localities A	B	C	D	F	G	Sum
Clethrionomys glareolus	92 (?)	54 (5)	35 (3)	21	3 (1)	–	205 (9)
Microtus agrestis	9	15	1	3	2	–	30
Microtus oeconomus	28	–	–	–	–	–	28
Apodemus sylvaticus	–	37 (1)	26	–	8 (1)	–	71 (2)
Apodemus flavicollis	–	25 (1)	–	–	–	–	25 (1)
Lemmus lemmus	35	–	–	–	–	6	41
Arvicola terrestris	–	–	–	–	1	–	1
Sorex sp.	17	21	4	–	10	–	52
Sum:	179 (2)	152 (5)	64 (3)	24	24 (2)	6	449 (12)

The number of examined animals is indicated.
The figures in parentheses represent the number of strains isolated.
See Fig. 1

TABLE 2. Yersinia enterocolitica *in Small Rodents. Individual Faeces Samples*

Locality	Date	Small rodent species	Total no. of individuals	Individuals harbouring Y.e. No.	%
A	July 1974	*Clethrionomys glareolus*	28	3	
		Microtus agrestis	5	0	
		Microtus oeconomus	10	3	
Sum			43	6	
C	July 1974	*Clethrionomys glareolus*	15	0	
		Apodemus sylvaticus	1	0	
Sum			16	0	
D	Aug 1974	*Clethrionomys glareolus*	28	0	
		Microtus agrestis	5	3	
Sum			33	3	
E	July 1974	*Clethrionomys rufocanus*	53	3	
		Clethrionomys rutilus	9	0	
Sum			62	3	
Total sum			154	12	8

Prior to sampling the traps were cleaned and then autoclaved or washed with 70 per cent alcohol. Animals were dissected sterilely immediately after death. Intestinal contents from ileum and rectum-colon were rubbed into a swab and stored on a modified Stuart transport medium (17).

Cultivation was performed within four days on Drigalski agar and *LSU* agar. Enrichment was carried out in selenite broth. The broth was incubated at 37 C for 18–22 hours after which subcultures were made on desoxycholate citrate agar and LSU agar. The broth was further incubated at

room temperature for five days and additional subcultures were made on the same media. All plates were incubated at 37° C for 18–22 hours and then stored overnight at room temperature. In this part only colonies with a morphology similar to Y.e. were considered. Pure cultures were made on lactose agar and then tested for urease activity in an urea-indol tube. Cultures showing a positive test were submitted to the same primary biochemical characterization as described in Part I.

III Serology

The isolated strains were tested by slide agglutination against O-antisera 1–34 (19) prepared against type strains at the National Institute of Public Health, Oslo, Norway. The O-antisera were prepared as described by *Wauters* (18). In the case of positive reaction the sera were titrated and tested by tube agglutination against the respective strains. A titer of 160 or higher was considered indicative of antigenic relationship of the isolated strain to that particular serotype. All tube agglutinations were performed with alcohol treated O-antigen preparations. Preliminary absorptions were carried out using the micro method described by *Kristiansen* (11).

Multiple isolations from the same faeces sample were considered identical if they reacted similarly on slide agglutination.

Plasma from animals harbouring Y.e. were tested by tube agglutination against homologous strains. Strains isolated from pools were agglutinated against plasma from each member of the pool. Blood samples were not available from the animals trapped in area F (Åsen).

IV Biotyping

All strains were submitted to the following additional biochemical tests:

Fermentation of carbohydrates (0.5 per cent solution): Adonitol, aesculin, arabinose, arbutin, cellobiose, dextrin, dulcitol, galactose, glycerol, inositol, inulin, levulose, malonate, maltose, mannitol, mannose, melibiose, raffinose, rhamnose, salicin, sorbitol, sorbose, starch, sucrose, trehalose and xylose. The strains were tested for both fermentative and oxidative metabolism of lactose (9).

The presence of the following enzymes were examined as described by *Le Minor* (14): Arginine dihydrolase, ornithine decarboxylase and lysine decarboxylase (Falkow medium without peptone), oxidase (Kovacs), nitrate reductase (tested in mannitol-motility tube), gelatin hydrolase (by means of photographic film) and β-galactosidase. Tests supplementary to the above included Voges-Proskauer (at 37° C and room temperature), plate test for lecithinase (5), methyl-red test and citrate utilization (Simmons medium).

Incubation was carried out at 37° C unless otherwise stated. The tests were read daily for four days. The strains were classified according to *Wauters* (18).

RESULTS

A total of 24 strains of *Y. enterocolitica* were isolated from 551 small rodents. Pooled faeces samples from 397 small rodents yielded 12 strains (Table 1). Individual faeces samples from 154 small rodents were cultured separately. Twelve (8 per cent) of these animals harboured Y.e. (Table 2). Each sample contained only one single strain with the exception of one of the pools from which two serologically different strains were isolated. No isolations were made from 52 shrews represented by pooled faeces samples.

Y.e. was isolated from six of the nine small rodent species (Table 1 and 2) and from six of the seven localities (Fig. 1) examined. The serological and biochemical classification of the strains are presented in Table 3. All but two strains showed antigenic relationship to known O-serotypes. The final serological typing is still under progress. A few deviations from the biotyping scheme proposed by *Wauters* are listed in the table.

When considering the pathogenicity of the isolated strains to the hosts, the following preliminary observations have been made: None of the animal sera showed any appreciable titer against the homologous strains. Autopsy has been performed in only a few instances. No macroscopic pathological changes were found.

DISCUSSION

Y. enterocolitica has frequently been documented as a cause of human infection in Norway, Sweden and Finland (1, 13, 16). The results of the present study indicate that Y.e. also has a widespread distribution among small rodents in this region having been isolated from several species of this animal group captured in various biotopes from six study areas. From Finse, the only locality where Y.e. was not found, only six animals were examined.

TABLE 5 *Yersinia enterocolitica* in *Small Rodents from Six Localities in Norway Sweden and Finland Biotypes and Relationship to Known O-Serotypes*

Locality	Date	Small rodent species	Biotype	Relationship to known O-Serotypes§
	Mar 1974	*Clethrionomys glareolus*	1	6
	Apr 1974	*Clethrionomys glareolus*	1	6
	July 1974	*Clethrionomys glareolus*	1	6
A	July 1974	*Clethrionomys glareolus*	1	6
	July 1974	*Clethrionomys glareolus*	1	6
	July 1974	*Microtus oeconomus*	1	5
	July 1974	*Microtus oeconomus*	1	7
	July 1974	*Microtus oeconomus*	3	–†
	Dec. 1973	*Apodemus sylvaticus*	1	16
	June 1974	*Clethrionomys glareolus*	1	†
B	June 1974	*Clethrionomys glareolus*	1	16
	June 1974	*Clethrionomys glareolus*	1‡	4
	June 1974	*Apodemus flavicollis*	1	1
	Oct. 1973	*Clethrionomys glareolus*	1	4
C	Apr 1974	*Clethrionomys glareolus*	1‡	4
	Apr 1974	*Clethrionomys glareolus*	1	6
	Aug 1974	*Microtus agrestis*	2	1
D	Aug. 1974	*Microtus agrestis*	2	3
	Aug 1974	*Microtus agrestis*	1	3
	July 1974	*Clethrionomys rufocanus*	1	6
E	July 1974	*Clethrionomys rufocanus*	1‡	4
	July 1974	*Clethrionomys rufocanus*	1ɔ	6
F	June 1974	*Clethrionomys glareolus*	1	6
	June 1974	*Apodemus sylvaticus*	1	1

* According to *Wauters* (18)
§ According to *Wauters et al.* (19)
† No relationship to serotype 1–34
‡ Attack lactose by fermentation.
ɔ Indole negative.

In Czechoslovakia *Aldová* & *Lim* investigated two species of small rodents (*Microtus arvalis* and *Apodemus sylvaticus*) and found that both were carriers of Y.e. (2) In the present study nine species of small rodents were examined. Six of these species harboured Y.e. No isolations were made from *Lemmus lemmus Clethrionomys rutilus* and *Arvicola terrestris* The last two species, however are only represented by nine and one individual, respectively The findings of these investigations indicate that most species of small rodents can harbour Y e.

It might also be expected that shrews, which occupy the same habitats as the rodents in question, could be carriers of Y.e. In this work, however no isolations were obtained from the shrews examined. A more extensive investigation of this animal group will be carried out at a later stage.

It was realized that the skill of distinguishing Y.e. from other organisms on the agar plates improved throughout the first part of the study During this part pooled faeces samples were used (Table 1) Hence, no conclusions can be made from this part other than those reporting the occurrence of Y.e. from various species and localities.

Based on the data from the second part of this work the frequency of animals harbouring Y.e. was found to be approximately 8 per cent (Table 2). No direct comparison can be made with the results of *Aldová & Lim* due to differences in the methods. Furthermore there is no information on the population densities of the small rodents in their study. The present work includes results from pre-peak and peak years. The high population densities during these years could facilitate the spread of bacteria within the populations. It is therefore possible that the frequency of animals harbouring Y.e. reaches a maximum during peak years. No definite conclusions, however, can be made until samples from various stages of the population cycle are analyzed.

The present findings include strains with antigenic relationship to serotype 1, 3, 4, 5, 6, 7 and 16 (Table 3). Four of these serotypes, namely 3, 5, 6 and 7, have not previously been recorded from small rodents. *Aldová & Lim* reported serotype 1, 2a, 2b-3, 4, 8, 8-19, 10, 16 and 28 from small rodents in Czechoslovakia. They also concluded that each study area was characterized by a predominating serotype. In the present work 42 per cent of the strains isolated were antigenically related to serotype 6. This serotype dominated in the Vassfaret area. The study, however, is too limited to allow similar conclusions for the other localities.

Most of the isolated strains showed antigenic relationship to serotypes previously reported from untreated drinking water in Norway (12). It seems likely that the drinking water may have become contaminated from small rodents.

Presently it is impossible to make definite conclusions concerning the pathogenicity of the isolated strains to the small rodent hosts. One possible assumption is that the isolated microbes can be opportunistic in small rodents. Latent infections may become manifest under conditions of stress such as occur during the population peak and the subsequent decline of small rodents in the northern ecosystems.

It is also possible that the isolated strains are a normal element in the intestinal flora of small rodents. It would appear that certain serotypes of Y.e. are pathogenic for only a narrow range of species (18). Small rodents may therefore serve as healthy carriers for strains specifically pathogenic for other species. For example serotype 1 has been isolated from enzooties among chinchillas (5, 6). In the present work strains antigenically related to this serotype were isolated from three localities and from three different species of small rodents (Table 3). However, these strains differed from the chinchilla pathogens by belonging to biotype 1 and 2. Likewise, serotype 3 is frequently isolated from human infections with Y.e. (1, 15, 16, 18). This serotype has also been isolated from animals including both healthy and diseased swine (7, 8). Two of the strains reported here showed antigenic relationship to serotype 3, but differed biochemically from the strains isolated from man and swine.

Presently it is not known to what extent the strains reported in this work can be pathogenic for man or animals.

In the present study the rodents were captured in the vicinity of holiday cabins and farms, within recreational areas and pastures, thus enabling Y.e. to be spread among man and domestic animals. Further investigation should therefore be carried out to clarify the reservoir function of small rodents in this yersiniosis.

I want to thank dr. *Tor Omland*, director of the Norwegian Defence Microbiological Laboratory, for his encouragement and for the facilities provided at his laboratory. Financial support was provided by the *Norwegian Defence Microbiological Laboratory*, the *Zoological Institute, University of Oslo* and the *Nordic Council for Terrestrial Ecology*.

REFERENCES

1. *Ahvonen P.*: Human yersiniosis in Finland. I. Bacteriology and serology. Ann. clin. Res. 4: 30–38, 1972.
2. *Aldová E. & Lim D.*: *Yersinia enterocolitica* in small rodents. Communication I: Pilot

study in two wildlife areas. Zbl. Bakt., I Abt. Orig A *226* 491–496, 1974

3 *Becht H*: Untersuchungen über die Pseudotuberculose beim Chinchilla. Dtsch. tierärztl. Wschr *69* 626–627 1962.

4 *Botzler R G., Wetzler T F & Cowan A.B.* *Yersinia enterocolitica* and Yersinia-like organisms isolated from frogs and snails. Bull. Wildl Dis. Ass. *4* 110–115 1968.

5. *Cruickshank R.* Medical microbiology A guide to the laboratory diagnosis and control of infection. 11 ed. E. & S Livingstone Ltd., Edinburgh and London 1968, p. 839–840.

6. *Daniels J J H M* Untersuchungen an als *Pasteurella pseudotuberculosis* diagnostizierten Stämmen on Chinchillas. Zbl. Vet. Med. *10* 413–419 1963

7 *Dickinson A. B & Mocquot G* Studies on the bacterial flora of the alimentary tract of pigs. I Enterobacteriaceae and other Gram-negative bacteria. J appl. Bact. *24* 252–284 1961

8. *Esseveld H & Goudzwaard C.*, On the epidemiology of *Y enterocolitica* infections Pigs as the source of infections in man. In Winblad, S. (Ed.) Contributions to microbiology and immunology vol. 2. *Yersinia, Pasteurella and F ... ella*. Karger Basel 1973 p. 99–101

9 *Hugh R. & Leifson E.* The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram negative bacteria. J Bact. *66* 24–26, 1953

10. *Juhlin I & Ericson C* A new medium for the bacteriologic examination of stools (LSU agar) Acta path. microbiol. scand. *57* 185–200 1961

11 *Kristiansen F.*, En modifikasjon av teknikken ved absorbsjon av mikrocellesera (mikrometode) VIII Nordiska veterinärmøtet Sekt. A, rapport 2 1–4 1958.

12. *Larsen J.*, *Yersinia enterocolitica* in drinking-water Scand. J Infect. Dis. *4*: 125–127 1972

13 *Larsen J & Bakkhus, Ø.*, *Yersinia enterocolitica*-infeksjoner hos mennesker T norsk Lægeforen. *91* 633–640 1971

14 *Le Minor L.* Le diagnostic de laboratoire des entérobactéries. 3 ed. Éditions de la Tourelle, St. Mandé 1967 p. 153–185

15 *Mollaret H H.*, L'infection humaine à *Yersinia enterocolitica* en 1970 à la lumière de 642 cas récents. Path. et Biol. *19* 189–205, 1971

16. *Nilehn B.* Studies on *Yersinia enterocolitica*. With special reference to bacterial diagnosis and occurrence in human acute enteric disease. Acta path. microbiol. scand. suppl. 206 1–48, 1969

17 *Ringertz O* A modified Stuart medium for the transport of gonococcal specimens. Acta path. microbiol scand. *48* 105–112, 1960.

18. *Wauters G* Contribution a L étude de *Yersinia enterocolitica*. Thesis. Vander Louvain 1970.

19 *Wauters G Le Minor L., Chalon A M & Larsen J* Supplément au schéma antigénique de *Yersinia enterocolitica*. Ann. Inst. Pasteur *122* 951–956, 1972.

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COMPARISON OF NEUTRALIZING AND IMMUNOPRECIPITATING ACTIVITY IN GUINEA PIG ANTISERA AGAINST HERPES SIMPLEX VIRUS TYPES 1 AND 2

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Jeansson, S & Vestergaard, B. F Comparison of neutralizing and immunoprecipitating activity in guinea pig antisera against Herpes simplex virus types 1 and 2. Acta path. microbiol. scand. Sect. B, 83: 343–346, 1975

Guinea pig antisera against Herpes simplex virus (HSV) types 1 and 2 were compared in neutralization tests and crossed immunoelectrophoresis. It was found that the HSV type specific neutralization of guinea pig antisera could be associated with the precipitating activity to HSV type 1 and 2 glycoproteins.

Key words Herpes simplex type 1 & 2 guinea pig antisera neutralizing and immunoprecipitating activity

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In a previous study (12) of Herpes simplex virus (HSV) type 1 and type 2 antigens by crossed immunoelectrophoresis, it was possible to identify several common and type specific antigens. Some of these antigens were identified as glycoproteins (14) *Powell et al.* (5) have recently reported the isolation of a glycoprotein antigen This antigen showed type specific reactions in complement fixation (CF) and neutralization tests (NT)

It has been shown that HSV types 1 and 2 antisera of high specificity in NT can be produced in hyperimmunized guinea pigs (3) In the present study neutralizing and precipitating activity of the guinea pig antisera were compared.

MATERIALS AND METHODS

The production of hyperimmune HSV type 1 and 2 guinea pig antisera and the plaque neutralization test used has been described previously (3) Briefly in the NT constant dose of virus and a varying dose of antiserum were used. The plot of log per cent surviving virus against the quantity of antiserum yielded straight line The relative potency of each serum is indicated by the numerical value of the slope (K value) of the straight line. The relative specificity of each antiserum can be expressed as the ratio of the slopes of lines (10)

The production of standard HSV type 1 and 2 antigens and a standard anti HSV type 1 and 2 rabbit IgG preparation used in crossed immunoelectrophoresis has been described previously (12) The test was performed on 10 × 10 cm glass plates covered with 1.5 mm thick agarose gel (Litex A GS-091) dissolved in tris/barbital buffer

pH 8.6 ionic strength 0.005 with 1 per cent Triton X 100 and 0.1 per cent sodium azide.

1 Dimension 15 µl of the standard HSV type 1 or 2 antigens was electrophoresed for 1 hour applying 10 volt per cm gel.

2. Dimension contained 6 µl of the standard anti-HSV type 1 and 2 antibody preparation per cm² gel and was electrophoresed for 16 hours, applying 1.5 volt per cm gel. The intermediate gel technic, used for the qualitative and quantitative analysis of precipitating antibodies, has been described by *Axelsen and co-workers* (1) In this study the intermediate gel contained 15 µl per cm² gel of the guinea pig antisera to be tested.

RESULTS

The results of plaque neutralization tests are shown in Fig 1 Both HSV type 1 and 2 guinea pig antisera showed a significantly greater capacity to neutralize homologous than heterologous virus. The ratio between K values for homologous and heterologous virus for anti HSV type 1 and 2 serum were 4.3 and 3 4 respectively

Crossed immunoelectrophoresis (Fig. 2) demonstrated that the precipitating activity of the guinea pig anti HSV type 1 serum was directed against three antigens (designated 3 6 and 11 (12) in the HSV type 1 preparation (plate C) and against two antigens in the HSV type 2 preparation (3 and 11 plate D) The activity of the anti HSV type 2 serum was directed against antigens 3 and 11 in the HSV type 1 preparation (plate E) and against 3 4 10 and 11 in the HSV type 2 preparation (plate F)

The precipitating antibody activity against antigen 11 was approximately ten times greater in the anti HSV type 1 serum than in the anti HSV type 2 serum (plates C and E) (1) The activity against antigen 3 in HSV type 1 and 2 antigen preparation was nearly the same for both antisera (plates C, D, E and F) (1) Type specific antibody activity was found against antigen 6 in the HSV type 1 antigen preparation (plates C and E) Type specific antibody activities against antigens 4 and 10 were found in the HSV type 2 antigen preparation (plates D and F)

In addition plates C, D E, and F show that a number of HSV type 1 and type 2 antigens which were detected by the standard antiserum from rabbits, did not react with antiserum from guinea pigs.

The reproducibility of these findings was documented in repeated experiments with three different batches of HSV type 1 and type 2 antigens.

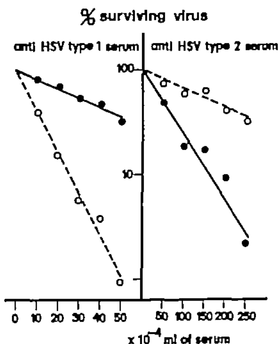


Fig 1 Multiplicity analysis of neutralizing potency of anti HSV type 1 and type 2 serum The neutralization tests were performed as described under "Materials and methods" Each point is an average for triplicate tissue culture tubes. The same virus preparations were used in all tests.
-- o -- o -- neutralization of HSV type 1
—•—•— neutralization of HSV type 2

DISCUSSION

Studies by *Roizman et al.* (6 7 8, 9) have shown that HSV induced glycoproteins incorporated in the plasma membrane and in the virus envelope are antigenically identical. Many of the HSV antigens demonstrated by crossed immunoelectrophoresis of detergent solubilized HSV infected cells are derived from the cell membranes. Another study (14) showed that antigens called 3 6, 10 and 11 were glycoproteins capable of binding with

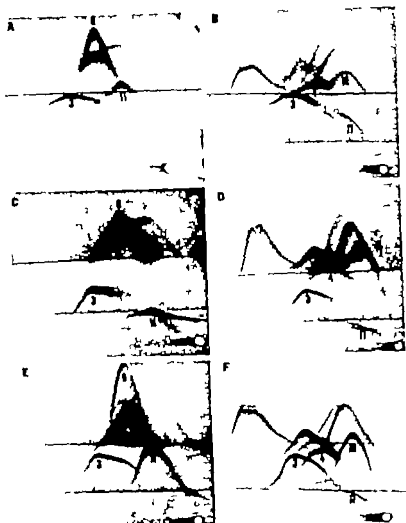


Fig 2 Crossed immunoelectrophoresis with guinea pig anti HSV type 1 and 2 sera in the intermediate gel. Antigen: HSV type 1 in plates A, C & E. HSV type 2 in plates B D & F Antisera in intermediate gel: Normal guinea pig in plates A & B. Anti HSV type 1 in plates C & D. Anti HSV type 2 in plates E & F

concanavalin-A. Recently three independent studies have shown that antibodies specifically directed against HSV glycoproteins were able to neutralize the virus (2 4 5) Thus a firm correlation seems to be established between antibodies to HSV glycoproteins and neutralizing antibodies. Guinea pig antisera against HSV show highly type specific reactions in neutralization tests (3) Our finding, that these guinea pig antisera almost exclusively reacted with HSV glycoproteins in immunoprecipitation, further substantiates that antibodies against glycoprotein antigens play a major role in neutralization. As compared to rabbit HSV antisera, guinea pig HSV antisera react with a limited number of antigens. The restricted reactivity of guinea pig antisera in crossed electrophoresis, and the reproducible precipitin patterns constitute a base for valid comparison with the results of the neutralization test.

Savage et al. (10) prepared immunoadsorbent gels of HSV antisera and showed that the different adsorption of HSV type 1

and type 2 glycoproteins to such gels was related to the specificity exhibited by the HSV antisera in neutralization tests. In our study we found both quantitative and qualitative differences in the HSV glycoprotein precipitin patterns exhibited by the two guinea pig antisera. The quantitative differences were found mainly in the activity against antigen 11 as the precipitating activity against that antigen was approximately ten times greater in the anti HSV type 1 guinea pig serum than in the anti HSV type 2 guinea pig serum. The qualitative differences were found in the activity against the HSV type specific antigens, only the anti HSV type 1 serum reacted with antigen 6 and only the anti HSV type 2 serum reacted with antigen 4 and 10 The almost identical activity of both antisera towards antigen 3 supports the previous finding that this antigen is common to both herpes virus types (12) The finding of one largely type 1 specific and two type 2 specific HSV antigens reacting with the guinea pig antisera supports previous results with HSV type 1 and type 2 specific guinea pig antisera produced by antibody suppression (3)

Crossed immunoelectrophoresis has shown that antigen 6 only is present in HSV type 1 preparations and that antigens 4 and 8 only are present in HSV type 2 preparations (12) It is therefore possible that the specific reactivity with these HSV type specific antigens exhibited by the guinea pig antisera can be correlated to the findings in the neutralization test. Monospecific antisera can be produced against HSV antigens by immunization with the corresponding precipitates (11 13) Such experiments, using antigens 4 6 and 10 are currently in progress to see if these antigens are responsible for the formation of HSV type specific neutralizing antibodies.

REFERENCES

1 *Axelsen H* Intermediate gel in crossed and fused rocket immunoelectrophoresis. In Axelsen, N H., Krøll, J & Weeke, B. (Eds.) A manual of quantitative immunoelectrophoresis. Scand. J Immunol. 2 Suppl 1 71–77 1973.

2 *Honess R W & Watson D H* Herpes simplex virus-specific polypeptides studied by polyacrylamide gel electrophoresis of immune precipitates. J Gen. Virol. 22 171 183, 1974

3 *Jeansson S:* Production of Herpes simplex antisera with increased type specificity in guinea pigs by antibody suppression. Proc. Soc. Exp. Biol. Med. In press 1974

4 *Ponce de Leon M Hessle H & Cohen, G. H* Separation of Herpes simplex virus-induced antigens by concanavalin A affinity chromatography J Virol. *12* 766–774, 1974.

5 *Powell, K L. Buchan A Sim Caroline & Watson D H* Type-specific protein in Herpes simplex virus envelope reacts with neutralising antibody Nature *249* 360–361 1974.

6 *Roane P R & Roizman B.* Studies of the determinant antigens of viable cells. II. Demonstration of altered antigenic reactivity of Hep-2 cells infected with Herpes simplex virus. Virology 22 1–8 1964

7 *Roizman, B. & Spring S B.* Alteration in immunologic specificity of cells infected with cytolytic viruses. In Trentin, J J (Ed) Proceedings Conference on Cross Reacting Antigens and *Neoantigens.* Baltimore, Williams and Wilkins Co. 1967 pp. 85–96.

8. *Roizman, B.* Herpesviruses, membranes and the social behaviour of infected cells In: Proceedings of the 3rd International Symposium on applied and Medical Virology (Fort Lauderdale Fla.) St Louis, M Warren Green 1970, pp. 37–72.

9 *Roizman B.* The modification of immunological specificity and function of cellular membranes by herpes viruses. Transpl Proc *3* 1179–1183 1971

10. *Savage T Roizman B. & Heine J W* Immunological specificity of the glycoproteins of Herpes simplex virus subtypes 1 and 2 J Gen. Virol. *17* 31–48, 1972.

11 *Watson, D H & Wildy P* The preparation of monoprecipitin antisera to herpes virus specific antigens. J Gen. Virol. *4* 163–168, 1969

12. *Vestergaard B F* Crossed immunoelectrophoretic characterization of Herpes virus hominis type 1 and 2 antigens Acta path. microbiol. scand. Sect B, *81* 808–810, 1973.

13 *Vestergaard B. F* Production of antisera to a Herpes simplex virus type 2 specific antigen Scand. J Immunol. In press. 1974

14 *Vestergaard B F & Bøg-Hansen T* Crossed immunoaffinoelectrophoresis of Herpes simplex virus type 1 and 2 antigens Scand. J Immunol. In press. 1974

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TRANSPLANTABLE HAMSTER TUMORS INDUCED WITH THE BK VIRUS

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Näse, L. M., Kärkkäinen, M. & Mäntyjärvi, R. A. Transplantable hamster tumors induced with the BK virus. Acta path. microbiol. scand. Sect. B, 83 347–352, 1975

Two tumors induced by BK virus in hamsters of an inbred strain were serially transplanted by subcutaneous injection of tumor explants. Both the original tumors as well as the transplants grew as solid, localized, encapsulated tumors. Histologically tumors were classified as fusocellular fibrosarcomas. Polymorphic tumor tissue was characterized by an abundance of collagen fibers and multinucleated giant cells. Cell lines established from tumors contained nuclear T antigen which stained with sera from a number of tumor-carrying hamsters, but also with SV40 T antiserum. Virion antigens were not detected in these cell lines, and no virus was isolated when tumor extracts were inoculated in Vero cells.

Key words Transplantable hamster tumors; BK virus.

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BK virus is a new member of the SV40-polyoma subgroup of papovaviruses. It was first isolated from urine of a renal transplant patient (5) Transplantation was complicated by granulomatous obstruction of the ureter but the possible role of the virus in patient's condition remained obscure. Biophysical properties of BK virus are similar to those of the other members of the SV40-polyoma group It resembles polyomavirus in agglutinating human and guinea pig erythrocytes (8) but antigenically it is not related to polyomavirus and only remotely related to SV40 virus (12)

Another new member of the same virus-group is the JC virus. It came from a patient with progressive multifocal leukoencephalopathy (PML) a rare neurological illness (11) Like the BK virus JC virus is antigenically distinct, but distantly related to SV40 and BK viruses (12)

In addition to other similarities with SV40 and polyoma viruses, both BK and JC virus have also been shown to have oncogenic properties under appropriate experimental conditions. Tumors have been induced in new-born hamsters with intracerebral inoculation of JC virus (16) *In vitro* transformation of BHK21 cells was achieved with infection of BK virus (7) and BK virus also seemed to be able to induce tumors in hamsters *in vivo* (*Gardner* cited by *Major & DiMayorca* (7))

In a series of experiments of the oncogenicity of BK virus we were able to induce tumors in hamsters. In this report we describe some properties of two transplantable tumor lines derived from BK virus-induced tumors.

MATERIAL AND METHODS

Cell Cultures

Vero cells (Biocult Laboratories, Glasgow Scotland) were used to propagate BK virus. Cells were dispersed with 0.25 per cent trypsin and grown in BME diploid (Gibco, Grand Island, N Y) supplemented with 10 per cent calf serum and 5 per cent tryptose phosphate broth (Difco Laboratories Detroit, Mich.)

H 50 cells, a hamster cell line transformed with *SV40* virus (6) were obtained from Dr *Fred Rapp* Tumors were induced with H 50 cells in hamsters of an inbred strain (LSH Lakeview Hamster Colony New Field N J) in this laboratory A cell line was then established with trypsinization of a tumor of second hamster passage. This cell line, designated H 50/LSH2, was found to contain a similar intranuclear SV40 T antigen as the original H 50 cells.

Cell cultures of tumors were made by treating minced tumor tissue with 0.25 per cent trypsin and 0 1 per cent collagenase (Sigma Chemical Co., StLouis, Mo.) in Hanks salt solution for 20 minutes at room temperature. Detached cells were collected by decanting, washed three times with Hanks solution, and planted in 250 cm^2 plastic flasks (Falcon Plastics, Oxnard Ca., U.S.A.) Growth medium consisted of BME diploid with 10 per cent fetal calf serum and 5 per cent tryptose phosphate broth.

Virus

BK virus strain was obtained from Dr *Sylvia Gardner* Extracts of Vero cells infected with BK virus were prepared as described before (8) To concentrate virus for animal inoculations extracts were clarified by centrifugation at $9,500 \times g$ for 15 minutes, and then concentrated by pelleting the virus through 15 per cent sucrose at $105,000 \times g$ for 90 minutes. Pellets were resuspended to one-tenth of the original volume. Concentrated virus preparations were also made by pelleting virus from maintenance medium. Virus concentrates prepared by these procedures had hemagglutinating titers of 1/10,000 to 1/20 000 and infectivity titers of 10^7 $TCID_{50}$/ml when titrated in Vero cells.

Tumor Induction and Transplantation

Golden Syrian hamsters of the LSH strain less than 24 hours old were inoculated subcutaneously with BK virus. Hamsters surviving inoculation were inspected weekly for tumors. Tumor-carrying animals were anaesthetized with ether and blood was collected by cardiac puncture. Tumors were excised, and pieces of tumor tissue were fixed in 10 per cent formalin in neutral phosphate buffer for histological staining. For transplantation tumors were minced with scissors, and injected in 0.5 ml volumes subcutaneously in adult hamsters.

Histological Staining

Standard methods of hematoxylin-eosin and van Gieson staining were used.

Fluorescent Antibody (FA) Test

Cells were grown on cover slips, washed with 0.05 M phosphate-buffered saline, pH 7.6, air dried, and fixed in acetone for 10 minutes at room temperature The indirect FA technique was used. Fluorescein-isothiocyanate-labeled anti-hamster globulin was purchased from Hyland (Costa Mesa, Ca.) and antirabbit globulin from Wellcome Research Laboratories (Beckenham, England) SV40 T antiserum was obtained from hamsters carrying tumors caused by transplanted H 50 cells. A rabbit immune serum against BK virus with a hemagglutination inhibition titer of 1:12,000 was used to test cells for virion antigens.

Hemagglutination (HA) and Hemagglutination Inhibition (HI) Tests

These tests were carried out as described before (8) with the exception, that to remove nonspecific inhibitors sera were pretreated with potassium periodate instead of receptor destroying enzyme (13)

RESULTS

Tumor Induction and Transplantation

Fifty new-born hamsters of 7 litters were inoculated with BK virus as described in Methods. Six months after inoculation, when a total of 18 hamsters was still under observation, a hard nodule was detected at the injection site of one of the animals. Another tumor appeared in the same litter 9 months after inoculation, but no more tumors developed until the experiment was terminated after an observation time of 15 months. The two tumors, designated BKT 1 and BKT-2, grew slowly in size until the hamsters were killed about a month after the appearance of tumors. Both tumors were transplanted to adult hamsters as described in Methods. Tumors appeared in 3 of the 4 hamsters which had received BKT 1 cells and in 2 of the 3 hamsters which had received BKT-2 cells. Transplantations were carried on serially

Transplants of the BKT 2 tumor line had a faster growth rate than those of BKT 1 tumors appeared in 2 to 3 weeks after injection of BKT 2 cells, whereas the latent period of BKT 1 tumors was about 4 weeks. The BKT 1 line is now in 4th and the BKT 2 line in 5th subcutaneous passage.

Tumor Pathology

Both the original tumors and their serial transplants were examined for macroscopic appearance and for histopathology. Tumor carrying hamsters were killed when the tumor had grown to a size of 20 to 40 mm in diameter. The gross appearance of tumors was round or nodular and tumors were well encapsulated. Tumor tissue was whitish and solid, in some parts of a tumor almost cartilaginous in consistence. Parts of softer tissue as well as hemorrhagic necrotic areas were found in larger tumors. No metastatic growth of tumor was revealed by visual examination of liver, kidneys, lungs or spleen of tumor carrying animals.

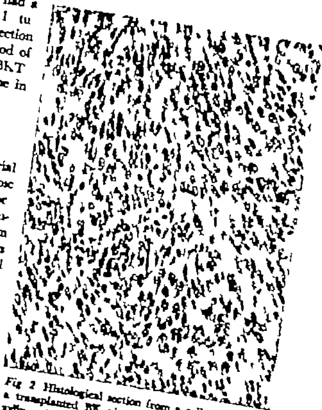
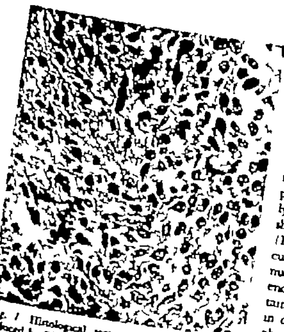


Fig. 2 Histological section from a cell rich area of a transplanted BK virus-induced tumor. Hematoxylin-eosin, × 195.



...g. 1 Histological section ... ...duced by BK virus. The p... ...ner ...odles of collagen fibers \ ... ...d by

Basic histopathological picture of both of the original tumors and their transplants was similar. Tumor tissue was polymorphic. Variations in cellularity were observed within the same tumor. The presence of thin collagen fibers, in some parts of a tumor as a thick net, was characteristic (Fig. 1). Collagen fibers and cells parallelly arranged between them often formed interlacing bundle like patterns. Necrotic areas were found frequently. The majority of tumor cells were spindle shaped with a relatively larger cytoplasm (Fig. 2). Their elongated nuclei had a reticular inner structure and several acidophilic nucleoli of various sizes. Giant cells were encountered frequently (Fig. 3). They contained several nuclei of various sizes located in different layers in the central part of an abundant cytoplasm. Mitotic figures, often multipolar were also common. No inclusion bodies could be detected. The tumors were classified as fusocellular fibrosarcoma...

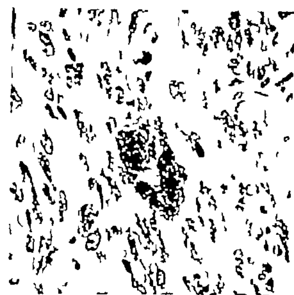


Fig. 3 Giant cell in a BK virus-induced tumor Van Gieson, × 330

Viral Markers in Tumor Cells

Attempts were made to isolate BK virus from 6 tumors. These included both the original tumors and transplanted tumors from 1st to 2nd hamster passage. Tumor samples stored at −80 C were frozen and thawed three times, and treated in a sonicator for 5 minutes (MSE, Surrey England) Suspensions were inoculated in cultures of Vero cells. Cell cultures were observed for three weeks. At the end of this period cells were disrupted with ultrasonication and tested for hemagglutinin with negative results.

All samples were passaged in Vero cells, and tested again for hemagglutinin after another three weeks, but no virus was isolated.

Three continuous cell lines, one established from BKT 1 and two from BKT 2 at 1st or 2nd hamster passage of the tumors, were tested for viral antigen using FA technique. No viral antigen could be detected with the rabbit immune serum which gave a strong positive reaction with human embryonic fibroblasts infected with BK virus.

The same cell lines were also tested for T antigen using both SV40 T antiserum and sera from hamsters carrying BK tumors. SV40 T antiserum reacted with a nuclear antigen present in all three cell lines (Fig. 4) The antigen was granular and filled the whole nucleus except the nucleoli the staining pattern was very similar to that shown by H 50/LSH2 cells with the same SV40 T antiserum.

Antibodies in the Sera of Tumor Hamsters

In sera of 6 tumor hamsters of the 16 tested so far antibody has been found which reacts both with H 50/LSH2 cells and with the BKT cell lines. Two of these sera were from the original tumor animals, the rest of them were from hamsters with transplanted BKT 2 tumors of 2nd and 3rd passage. The staining pattern shown by these sera was again similar to that produced by SV40 T antiserum.

In addition to the T antibody sera of the original tumor animals also contained HI antibodies against BK virus in titers of 1/640 and 1/2560. The rest of the tumor-carrying hamsters were antibody negative in HI test On the other hand, in the 3rd passage of



Fig. 4 T antigen in a cell line derived from a BK virus-induced hamster tumor Indirect immunofluorescent staining with antiserum against SV40 T antigen, × 330.

BKT 1 two hamsters without apparent tumors were killed 4 and 7 weeks after transplantation, respectively Sera of these animals were negative for T antibody but contained HI antibodies against BK virus in titers of 1/320 and 1/1280 All the sera positive in HI test were also tested in FA tests against human embryonic fibroblasts infected with BK virus, and found to give positive reactions.

DISCUSSION

Tumors which developed in hamsters after subcutaneous injection of BK virus were fibrosarcomas. Encapsulation and lack of metastatic growth of the BK tumors are properties also characteristic to subcutaneous hamster tumors induced with SV40 virus (2) In the same way the histopathology of BK tumors with spindle cells, collagen fibers and giant cells is similar to that of SV40 tumors (1)

It has been shown earlier that permissive cells infected with BK virus contain an intranuclear antigen, which crossreacts with SV40 T antigen (15) Our results show that hamster cell lines derived from BK virus-induced tumors also contain an antigen staining with SV40 T antiserum in the FA test. The cross-reactivity was found to be reciprocal, because some of the sera of BK tumor hamsters also stained SV40 tumor cells. A relationship has been detected by electron microscopic agglutination between virion antigens of BK and SV40 viruses (12) The relationship between the T antigens of BK, JC (16) and SV40 viruses seems, however to be much closer than the relationship between the capsid antigens of these viruses.

Restriction endonuclease analysis of the DNA of BK, JC and SV40 viruses revealed significantly different fragmentation patterns (10) It will be interesting to see if molecular hybridization studies will show identical sequences in the DNAs of these viruses long enough to code for a protein carrying T antigen specificity

Our attempts to isolate virus from BK tumor tissue have been negative. This may be due to the limited susceptibility of Vero cells to BK virus, but it is also possible, that our tumors do not release virus spontaneously Two of the brain tumors induced in hamsters with JC virus failed to produce virus, although virus was found in other tumors tested (16) We are in the process of testing if the cell lines established from BK tumors can be induced to release virus by fusion with susceptible cells. The appearance of HI antibodies in two hamsters which had received tumor cells but did not develope tumors, may be an indication of periodical release of virus from tumor cells.

Our observations confirm the earlier findings on the oncogenic potential of BK virus (7) Thus both of the new human papovaviruses, BK and JC virus, share the oncogenicity of SV40 and polyoma virus. It is characteristic to the latter two viruses, however that their oncogenicity can be brought out only with special procedures, *e.g.* by injecting large amounts of virus to new-born rodents. Neither SV40 nor polyoma virus seem to cause tumors under natural conditions despite of their wide-spread occurrence (3) Antibody surveys have revealed that BK virus is a common human infectious agent (4 9 14) It remains to be studied whether BK virus has any connection with human tumors, either benign or malignant, or whether its oncogenicity is limited to artificial conditions of laboratory experiments.

We express our thanks to *Aua Reijula* and *Paula Taskinen* for their competent technical assistance. This work was supported by the *Sigrid Jusélius Foundation*

REFERENCES

1 *Berman L, D* Comparative morphologic study of the virusinduced solid tumors of Syrian hamsters. J natn. Cancer Inst. 39: 847–901 1967

2. *Eddy B E.* Simian Virus 40 (SV-40): An oncogenic virus. Progr exp. Tumor Res. 4: 1–26 1964.

3. *Gardner M B., Henderson B. E., Menck, H., Parker J Estes J D & Huebner R.* Spon-

taneous tumors and C-type virus in polyoma-infected aging wild house mice. J natn. Cancer Inst. 52: 979–981 1974

4 *Gardner S D* Prevalence in England of antibody to human polyomavirus (B.K.) Brit. med. J *1* 77–78, 1973

5 *Gardner S D., Field A M., Coleman, D V & Hulme B.:* New human papovavirus (B.K.) isolated from urine after renal transplantation. Lancet *1* 1253–1257 1971

6. *Khera K S Ashkenazi A., Rapp F & Melnick J L.* Immunity in hamsters to cells transformed *in vitro* and *in vivo* by SV 40. J Immun. *91* 604–613 1963.

7 *Major E. O & DiMayorca G.*. Malignant transformation of BHK$_{21}$ clone 13 cells by BK virus—A human papovavirus. Proc. natn. Acad. Sci. U.S.A. *70* 3210–3212, 1973.

8. *Mäntyjärvi R. A Arstila P P & Meurman O H.* Hemagglutination by BK virus, a tentative new member of the papovavirus group. Infect. Immun. *6* 824–828, 1972.

9 *Mäntyjärvi R. A., Meurman O H., Vihma L. & Berglund B* A human papovavirus (B.K.) biological properties and seroepidemiology Ann. Clin. Res. *5* 283–287 1973

10. *Osborn J E. Robertson S M., Padgett B. L. ZuRhein G M Walker D L. & Weisblum B* Comparison of JC and BK human papovaviruses with simian virus 40 Restriction endonuclease digestion and gel electrophoresis of resultant fragments. J Virol. *13* 614–622, 1974

11 *Padgett B L., Walker D L., ZuRhein, G. M., Eckroade R. J & Dessel, B. H* Cultivation of papova-like virus from human brain with progressive multifocal leucoencephalopathy Lancet *1* 1257–1260, 1971

12. *Penney J B., Jr & Narayan O* Studies of the antigenic relationships of the new human papovaviruses by electron microscopy agglutination. Infect. Immun. *8* 299–300, 1973.

13. *Robinson R. Q. & Dowdle W R.* Influenza viruses. In Lennette, E. H., & Schmidt, N. J., (Ed.): Diagnostic Procedures for Viral and Rickettsial Infections. American Public Health Association, Inc., New York 1969, p. 414–433.

14 *Shah, K. V., Daniel R. W & Warszawski, R. M* High prevalence of antibodies to BK virus, an SV40-related papovavirus, in residents of Maryland. J Infect. Dis. *128.* 784–787 1973.

15. *Takemoto K. K & Mullarkey M F.:* Human papovavirus, BK strain Biological studies including antigenic relationship to simian virus 40. J Virol. *12* 625–631 1973.

16. *Walker D L Padgett B. L., ZuRhein, G. M Albert A E. & Marsh, R. F* Human papovavirus (JC) Induction of brain tumors in hamsters. Science *181* 674–676, 1973.

Acta path. microbiol. scand. Sect. B, 83 353–366, 1975

RADIORESPIROMETRIC STUDIES IN GENUS *NEISSERIA*

1 The Catabolism of Glucose

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Holten, E. Radiorespirometric studies in genus *Neisseria*. 1 The catabolism of glucose. Acta path. microbiol. scand. Sect. B, 83: 353–366, 1975

The catabolism of glucose in selected *Neisseria* species was studied by radiorespirometry The Entner-Doudoroff and pentose phosphate pathways were operating in all species, the greater part of the substrate being routed through the former somewhat dependent on the medium used. Acetate was oxidized via the tricarboxylic acid cycle. In all species, a fraction of the triose was recycled through fructose phosphates to glucose 6-phosphate. Phosphate inhibited the oxidation of acetate. In media devoid of phosphate and sodium (*N meningitidis* and *N gonorrhoeae*) or of phosphate and potassium (other saccharolytic *Neisseria*) the con er sion of triose to pyruvate was inhibited. In these media the catabolism of glucose proceeded slowly and no substrate was used for biosynthetic purposes. The results point to a difference in the regulation of glucose metabolism in pathogenic and non-pathogenic *Neisseria*.

Key words *Neisseria* glucose catabolism; radiorespirometry

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In *Neisseria meningitidis* glucose is metabolized by the Entner Doudoroff and pentose phosphate pathways (12) By determining the specific activity in pyruvate produced from labelled glucose *Jyssum & Jones* (15) found that 67–87.5 per cent of the administered glucose was metabolized by the Entner-Doudoroff pathway while the remaining 12.5–33 per cent was channelled through the pentose phosphate pathway The Embden-Meyerhof pathway played an insignificant role, although extracts from *N meningitidis* were shown to catalyze all the necessary reactions in this pathway (14)

It has further been found that all the "true neisserias" contain glucokinase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (4, 5) which catalyse the first steps of the pentose phosphate pathway These species also have 6-phosphogluconate dehydrase and 2-keto-3-deoxy-6-phosphogluconate aldolase, except *N cinerea* and *N elongata*, which lack the dehydrase (5) and should accordingly be able to metabolize glucose also by the Entner Doudoroff pathway

In the present study the glucose metabolism in strains of selected *Neisseria* species have been examined by radiorespirometry to compare the pathways of glucose degradation in the different species.

MATERIALS AND METHODS

Strains. The *Neisseria* strains have been used in previous experiments (5 8). Instead of the wild type of *N meningitidis* B 8152/66, which is non-saccharolytic, a glucose and maltose positive mutant of this strain (9) was used.

Media. The medium used in the radiorespirometric studies was essentially the minimal medium developed by *Jyssum* (10) except that glucose was omitted, and contained per litre K_2HPO_4 7 g KH_2PO_4 3 g $MgSO_4$ $7H_2O$ 0 1 g NH_4Cl 1.5 g $FeSO_4$ 0.001 g $MnSO$ 0.001 g $CaCl_2$ H_2O 0 1 g and $Na_2S_2O_3$ 0.025 g Since phosphate is known to inhibit the complete oxidation of glucose (14) it was also desirable to use phosphate-free medium. In some experiments the phosphates were therefore replaced by 0.15 M NaCl or KCl and the medium was buffered at pH 7 4 by 0.01 M Tris/HCl. In a series of experiments also Heart Infusion Broth (Difco) was used.

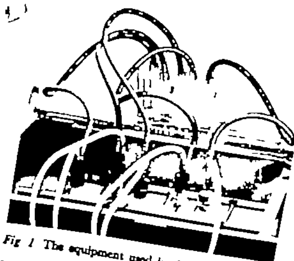
Cell suspensions Cells were grown on blood agar plates for 18–20 hours at 37 C in humid atmosphere with increased CO_2 content. The growth was harvested in 0.85 per cent saline, centrifuged and suspended in medium to a final concentration of 3 mg/ml (dry weight) A suspension with an O D = 1.0 at 490 nm was considered to contain 1 mg/ml (1)

Radiorespirometric equipment Because an apparatus for radiorespirometry was not available, a suitable set-up had to be devised (Fig. 1) It consisted of a number of 100 ml Erlenmeyer flasks in a water bath. The flasks were shaken linearly with 110 strokes/min. Air was blown through the flasks at a rate of 60–70 ml/min. From the flasks, the air was led through hypodermic needles (23 gauge) to the bottom of 16 × 160 mm test tubes containing the CO_2 trapping solution. The solution was changed at intervals simply by moving the needle from tube to tube.

Radiorespirometry was carried out as described by *Wang* (18) 10 ml of cell suspension was placed in each Erlenmeyer flask and stirred by shaking for 30 minutes at 37 C, after which 0.2 µCi of specifically ^{14}C-labelled glucose was added. The optimal amount of carrier glucose varied from strain to strain, and had to be determined in preliminary experiments. The CO_2 traps contained 10 ml of ethanolamine in ethanol (1:2 by volume) which was replenished every 10 minutes, or every 30 minutes in some experiments. 3 ml of the trapping solution was transferred to counting vials and mixed with scintillation fluid. The scintillation fluid was composed of 2 parts of 0.5 per cent 2,5-diphenyloxazole and 0.005 per cent 1,4-di-[2 (4-methyl 5-phenyloxazolyl)]-benzene in toluene, and 1 part of Triton X 100. Scintillation fluid without Triton X 100 was not found to give satisfactory results as it did not mix completely with even small volumes of the trapping solution, giving low and variable counting efficiency

After each experiment, the cells were separated from the medium by centrifugation, washed once in 0.85 per cent saline and suspended in water. Appropriate samples of cell suspension and of the combined washings and medium were placed in counting vials, the volume made up to 4.5 ml with water and counted after the addition of 10 ml of scintillation fluid. The recovery of radioactivity in CO_2, cells and medium was recorded as per cent of administered label. The data for carbon 4 of glucose was calculated from the results obtained from glucose-3-^{14}C and glucose-3 4-^{14}C (18)

Chemicals. D-glucose 1-^{14}C, D-glucose 2-^{14}C, D-glucose-3-^{14}C, D-glucose-3 4-^{14}C and D-glucose-



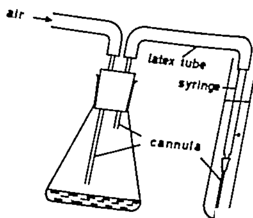


Fig 1 The equipment used in the study *Left* overall view *Right* schematic drawing.

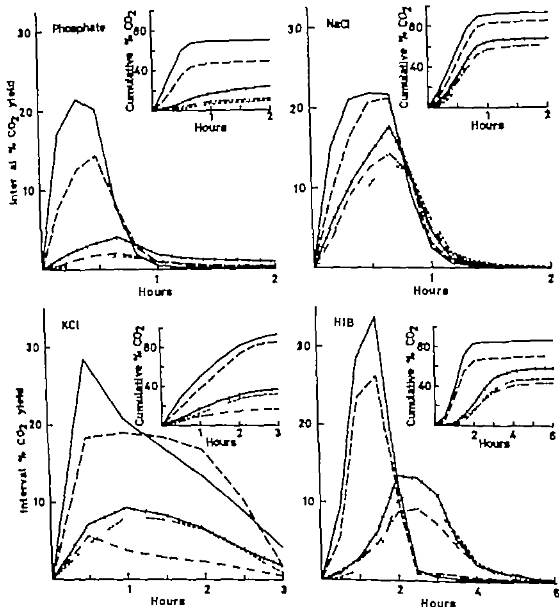


Fig 2 Radiorespirometric pattern for the utilization of glucose by *N meningitidis* M 6. *Phosphate* Phosphate-based minimal medium. *N Cl:* NaCl/Tris-based minimal medium. *KCl:* KCl/Tris-based minimal medium. *HIB* Heart Infusion Broth. Glucose-1-^{14}C ——— glucose-2-^{14}C —|—|—|—; glucose-3-^{14}C: – – – – glucose-4-^{14}C — — —; glucose-6-^{14}C - - - -.

6-^{14}C were purchased from New England Nuclear Corp., Boston, Mass., U.S.A. Scintillation fluid was obtained as a ready-to-use solution made by Ing. F Heidenreich, Oslo, Norway from chemicals provided by Koch-Light Laboratories, Ltd., Colnbrook, Buckinghamshire, England.

RESULTS

The radiorespirometric patterns are shown in Figs. 2–7 and the isotopic recovery at the end of each experiment in Table 1

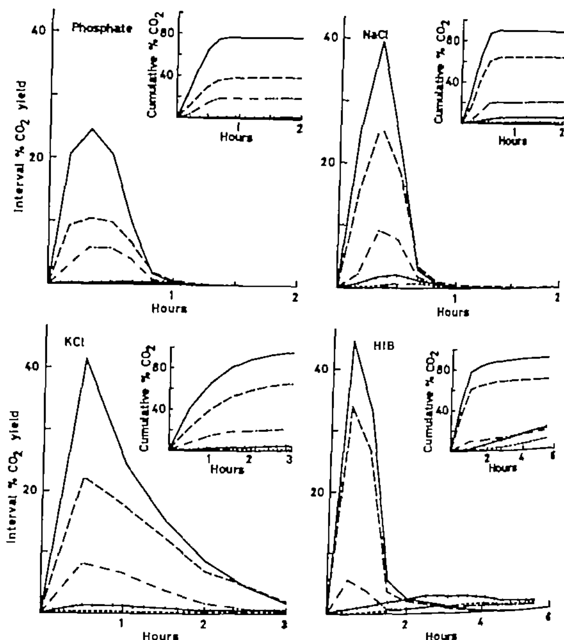


Fig 3 Radiorespirometric pattern for the utilization of glucose by *N gonorrhoeae* 1 a. Symbols as in Fig. 2

1 N meningitidis *and* N gonorrhoeae

Carbons number 1 and 4 of glucose (C1 and C4) are converted to CO more extensively than the other carbons. This indicates that the substrate is catabolized mainly via the Entner Doudoroff pathway A part of it is also catabolized via the pentose phosphate pathway as the CO_2 yield from C1 exceeds that from C4 The acetate appearing from the decarboxylation of pyruvate is oxidized via the tricarboxylic acid cycle since the yield from C2 is larger than that from C3 (18)

During the early phase of the experiments there is a preferential oxidation of C6 over C3 which suggests a recycling of trioe through fructose 1,6-diphosphate and fruc

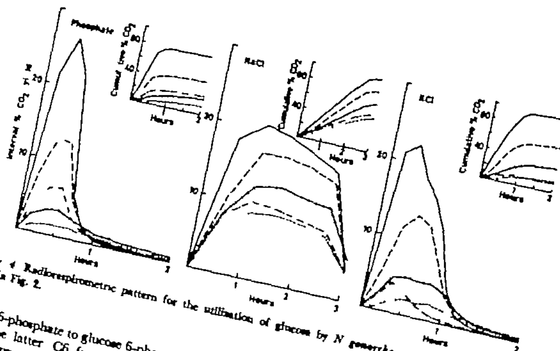


Fig. 4. Radiorespirometric pattern for the utilization of glucose by *N gonorrhoeae* 21308/70. Symbols as in Fig. 2.

tose 6-phosphate to glucose 6-phosphate (21). In the latter C6 from the original glucose will appear in position 1. The catabolism of this glucose 6-phosphate will then yield CO from the original C6 of glucose.

The radiorespirometric pattern and CO_2 yield depend upon the medium used.

Heart Infusion Broth. In *N meningitidis* M6 and *N gonorrhoeae* 1a the CO_2 evolution from C2, C3 and C6 reaches its maximum when the glucose has been consumed and the production of CO from C1 and C4 has ceased. This delay was much less pronounced in *N meningitidis* Ne 15 and B 8157/66 (not shown). In the minimal media, the peak CO production from all carbons is almost coincident, with some variation which will be discussed below.

In *N gonorrhoeae* 1a (Fig 3) the curve for C6 is biphasic with an interval CO yield of 0.80 per cent at 1½ hours and 1.16 per cent at 4 hours. The first peak most likely comes from recycling of triose (see above) and the second one from conversion of triose to pyruvate according to the usual Embden-Meyerhof scheme with subsequent decarboxylation, and oxidation of the acetate via the tricarboxylic acid cycle.

Phosphate-based medium. The CO_2 yield is reduced compared to that obtained using other media, indicating a less complete oxidation of glucose. Because of the low CO yield from C2, C3 and C6 most pronounced in *N meningitidis* M 6 also the oxidation of acetate seems to be diminished. The ratio C1/C4 is increased, meaning that a larger part of the substrate is shunted through the pentose phosphate pathway.

NaCl/Tris-based medium. In this medium glucose is oxidized rapidly and, as regards *N meningitidis* M 6 and *N gonorrhoeae* 21308/70 more completely than in the other media.

KCl/Tris-based medium. The metabolism of glucose in this medium proceeds slowly visualized by the gradual decrease in interval CO yield from C1. In the other media, the CO production from this carbon ceases abruptly even in the presence of a large amount of substrate which only makes the peak of the curve broader (not shown).

In *N meningitidis* M 6 and *N gonorrhoeae* 21308/70 there is somewhat less incorporation of label into cellular components, and hence a larger fraction of substrate used for energy production than that found in other media.

In these two strains, the peaks of the curves for C1 and C6 are coincident and the C6 curve is far different from that of C3. From

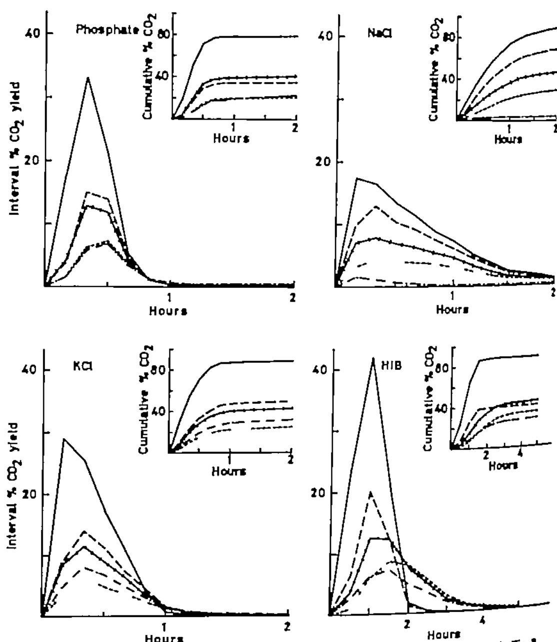


Fig 5 Radiorespirometric pattern for the utilization of glucose by *N. perflava* CN. Symbols as in Fig. 2.

C6 only 17 per cent and 12 per cent appear as CO_2, while 81 per cent and 91 per cent are excreted into the medium, in *N meningitidis* M 6 and *N gonorrhoeae* 21508/70, respectively. Probably far the greatest part of this CO_2 will be the result of recycling of triose. Because of the discrepancy between the C3 and C6 curves it is assumed that only a small amount of C6 can reach the acetate level. The total CO_2 recovery from C4 is not affected, but the peak CO_2 production from this carbon is delayed. These results suggest that in KCl/Tris-based medium, triose can not readily be converted to pyruvate. A part of it is recycled to hexose phosphates, C4 will be split off from most of the remainder to appear as CO_2, while the rest of the molecule is excreted back into the medium.

2 Other saccharolytic Neisseria

The other saccharolytic *Neisseria* species also catabolize glucose via the Entner Doudoroff and pentose phosphate pathways, and oxidize acetate via the tricarboxylic acid cycle (Figs. 5–7) The portion of the glucose to be shunted through the pentose phosphate pathway is larger than that otherwise seen in the case of the pathogenic species. *N perflava* ATCC 10555 and *N lactamica* ATCC 23970 deviate somewhat from the other strains, and are discussed below There are again some medium-dependent variations in the radiorespirometric patterns.

Heart Infusion Broth. The peak production of CO_2 from C2, C3 and C6 is much delayed in *N mucosa* M 4 In the other strains this delay is barely visible (results shown only for *N sicca* CN (Fig. 5))

Phosphate-based medium. In this medium one finds mainly the same pattern as that seen in the cases of *N meningitidis* and *N gonorrhoeae*

NaCl/Tris-based medium. The radiorespirometric pattern and CO_2 recovery data show that there is slow catabolism of substrate, CO_2 production from C4 is delayed as compared to C1 the C6 pattern resembles that of C1 (results shown only for *N sicca* CN (Fig 5)) and there is little incorporation of label into cellular components, the same results as those seen in KCl/Tris-based medium, using cells of *N meningitidis* and *N gonorrhoeae*

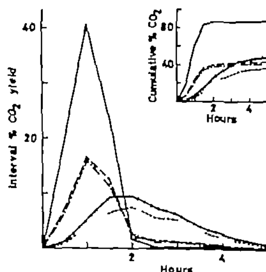


Fig 6 Radiorespirometric pattern for the utilization of glucose by *N perflava* ATCC 10555 in Heart Infusion Broth. Symbols as in Fig. 2.

KCl/Tris-based medium. Also in this medium the glucose tends to be catabolized somewhat slowly but less so than in the NaCl/Tris-based medium. The radiorespirometric patterns are otherwise comparable to those obtained using Heart Infusion Broth and phosphate-based medium.

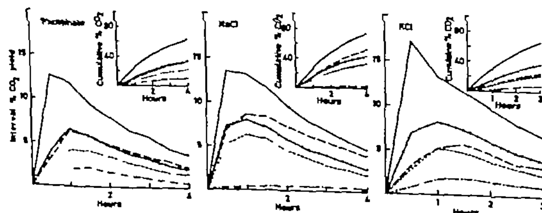


Fig 7 Radiorespirometric pattern for the utilization of glucose by *N lactamica* ATCC 23970. Symbols as in Fig. 2.

TABLE 1. *Incorporation of Specifically* ^{14}C

Species and strain	Labelled carbon	μmoles substrate	Phosphate-based medium: CO_2	Cells	Medium	Total
N meningitidis M 6	1	5	71	2	28	101
	2		30	8	58	96
	3		12	9	83	104
	4		50	6	46	102
	6		13	10	80	103
N meningitidis No 15	1	5	76	4	21	101
	2		39	22	43	104
	3		25	25	52	102
	4		39	18	44	101
	6		31	21	46	97
N meningitidis B 8152/66	1	5	67	3	27	97
	2		21	12	68	101
	3		11	14	75	100
	4		46	12	39	97
	6		19	14	66	99
N gonorrhoeae 1 a	1	6	79	1	26	106
	2		2	1	100	103
	3		1	1	105	107
	4		41	3	66	110
	6		21	3	83	107
N gonorrhoeae 21508/70	1	6	78	5	26	109
	2		22	14	69	105
	3		13	15	76	104
	4		45	14	41	100
	6		26	18	63	108
N sicca CN	1	2.5	81	8	10	99
	2		37	23	39	99
	3		23	25	32	100
	4		43	27	33	103
	6		27	28	44	99
N mucosa M 4	1	4	89	7	5	101
	2		44	25	22	91
	3		31	30	31	92
	4		40	29	25	94
	6		39	28	26	97
N perflava ATCC 10555	1	4	67	13	18	98
	2		17	22	54	93
	3		22	21	55	98
	4		35	25	56	96
	6		40	23	31	94
N perflava A 2	1					
	2					
	3					
	4					
	6					

…abelled Gluc… into CO_2, Cells and Medium

NaCl/Tris-based medium

µmoles substrate	CO_2	Cells	Medium	Total
5	94	3	3	100
	69	14	12	93
	37	19	22	98
	86	13	7	106
	63	19	18	100
8	92	1	4	97
	7	2	94	103
	2	1	108	111
	67	4	23	96
	23	4	75	102
1.5	97	2	4	103
	63	9	36	108
	43	9	47	99
	80	8	14	102
	49	11	40	100
1.5	92	0	3	95
	49	2	49	100
	32	2	69	103
	72	4	20	96
	5	4	92	101
2.5	96	0	4	100
	56	2	38	96
	45	3	56	104
	71	4	28	103
	32	4	67	103
2.5	44	2	58	104
	1	2	95	98
	2	2	96	100
	17	4	80	101
	16	4	80	100

KCl/Tris-based medium

µmoles substrate	CO_2	Cells	Medium	Total
5	93	0	7	100
	37	3	69	109
	32	5	71	108
	85	4	18	107
	17	4	81	102
8	93	1	7	103
	5	2	99	106
	1	3	100	104
	63	4	30	99
	21	0	75	96
2	97	0	8	105
	31	9	70	110
	14	8	77	99
	60	4	58	102
	12	6	91	109
1.5	89	6	4	99
	44	22	38	104
	28	24	49	99
	51	27	23	101
	33	31	37	101
2	71	11	22	104
	9	13	74	96
	15	14	70	99
	37	19	43	101
	37	20	44	101

Heart Infusion Broth

µmoles substrate	CO_2	Cells	Medium	Total
100	87	5	6	98
	39	18	19	96
	48	22	26	96
	71	13	10	94
	44	24	26	94
100	89	5	5	99
	35	22	43	100
	24	26	53	103
	71	18	1	90
	23	30	43	98
100	90	5	6	101
	34	23	40	97
	22	30	33	103
	61	20	8	89
	24	34	41	99
40	90	3	9	102
	21	7	70	98
	10	8	93	111
	70	7	10	87
	18	12	73	103
20	89	3	7	99
	45	17	29	91
	34	24	44	102
	41	18	46	83
	28	25	41	94
50	88	6	5	99
	56	23	12	91
	43	33	20	98
	49	24	12	85
	40	36	18	94
20	86	6	8	100
	48	17	32	97
	36	24	42	102
	43	29	18	90
	41	32	28	99
10	87	4	7	98
	47	19	27	93
	37	28	43	108
	31	26	24	81
	29	31	36	96

TABLE

Species and strain	Labelled carbon	μmoles substrate	Phosphate-based medium CO_2	Cells	Medium	Total
N flava ATCC 14221	1	2.5	89	2	6	97
	2		66	7	21	94
	3		56	9	33	98
	4		54	12	30	96
	6		39	14	41	94
N subflava ATCC 11076	1	4	78	4	15	97
	2		47	10	37	94
	3		38	11	48	97
	4		48	16	40	104
	6		42	10	41	93
N lactamica ATCC 23970	1	1	62	0	38	100
	2		33	6	60	99
	3		22	8	74	104
	4		33	9	67	109
	6		10	10	88	108

The CO_2 recovery data were recorded at the end of each experiment. The experiments were terminated 3–4 hours using minimal media.

3 *Deviating strains*

In *N perflava* ATCC 10555 using Heart Infusion Broth, one finds that the curves for C4 and C6 are near identical, their peaks being coincident with that of C1 while the peaks of C2 and C3 appear later (Fig. 6) Also using minimal media, the C4 and C6 curves are very similar Another feature is the preferred oxidation of C3 over C2 in all minimal media, and the very low overall CO yield in NaCl/Tris-based medium, indicating an incomplete oxidation of glucose in this medium.

In *N lactamica* the height and shape of the curves are not influenced by great variations in glucose concentration (0 05–20 μmoles) tested in the strains 1379 (not shown) and ATCC 23970 The rate of glucose transport across the cell membrane thus seems to be dependent on the extracellular concentration of glucose. Apart from this, the overall pattern seems to be consistent with that of the other non-pathogenic *Neisseria*.

4 *Non saccharolytic* Neisseria

Using glucose-1 ^{14}C of high specific activity very small amounts of $^{14}CO_2$ was evolved from *N flavescens* ATCC 13120 and *N elongata* M 2, but not from *N cinerea* 165/61. Attempts were made to alter the permeability of *N flavescens* cells by washing in distilled water prior to the experiment, with no effect on CO_2 production. Further attempts to investigate possible breakdown of glucose in these species were not done.

DISCUSSION

In all species, glucose is catabolized via the Entner Doudoroff pathway a lesser part of it also via the pentose phosphate pathway The pyruvate produced is rapidly decarboxylated to acetate, which is oxidized in the tricarboxylic acid cycle. Using complex medium (Heart Infusion Broth) there is a delay in the appearance of CO from C2, C3 and C6 in some strains. This is consistent with the assumption that acetate is incorporated into

tinued)

substrate	NaCl/Tris-based medium CO_2	Cells	Medium	Total	μmoles substrate	KCl/Tris-based medium CO_2	Cells	Medium	Total	μmoles substrate	Heart Infusion Broth CO	Cells	Medium	Total
5	93	0	4	99	0.5	98	2	3	103	15	96	2	3	101
	76	1	15	92		77	9	14	100		56	20	17	93
	70	2	25	97		68	13	24	105		44	28	29	101
	79	3	12	94		54	20	24	98		44	24	12	80
	11	4	88	103		51	19	26	96		39	30	24	93
	95	0	3	98	2	94	3	7	104	20	93	3	5	101
	53	1	42	88		71	10	23	104		60	16	19	95
	38	1	61	100		61	9	33	103		49	25	31	105
	88	1	13	102		79	17	19	115		50	19	16	85
	10	1	89	100		61	11	27	99		48	25	25	98
1	73	0	26	99	1	66	0	40	106					
	43	10	46	99		37	12	60	109					
	32	13	62	107		22	12	64	98					
	52	7	36	95		25	17	59	101					
	3	8	97	108		8	17	76	101					

at the cessation of the CO_2 evolution, usually after 3 hours using Heart Infusion Broth, and after

the pool of amino acids and other cellular components which are catabolized as the need for energy rises when the glucose has been consumed (20).

According to the present results, the tricarboxylic acid cycle seems to be as active in *N. meningitidis* and *N. gonorrhoeae* in which in vitro malate dehydrogenase activity has not been found (9, 11), as in the other saccharolytic *Neisseria* species. The only exception is *N. gonorrhoeae* 1 a in which the oxidation of acetate proceeds very slowly and incompletely, possibly because of inefficient synthesis of citrate (6).

Triose appearing from the Entner-Doudoroff cleavage of 2-keto-3-deoxy-6-phosphogluconate will follow the usual Embden-Meyerhof scheme to pyruvate. The oxidation of the pyruvate would give a C6 curve differing little, if at all, from that of C3. However, the preferential oxidation of C6 over C3 during the early phase of the experiments implies that triose will also be recycled to hexose phosphate in all species. This is supported by the recent report of *Morse et al.* (17) who suggest that recycling of triose occurs in *N. gonorrhoeae*. *Jyssum* (13) found rapid incorporation of label from glucose-6-[14]C into pyruvate, using resting cells of *N. meningitidis*. In an experiment with cells suspended in phosphate buffer 5.3 per cent of the activity of pyruvate was located in the carboxyl carbon (15), indicating conversion of triose to pyruvate according to the Embden-Meyerhof scheme, as well as recycling of a fraction of the triose. It has been pointed out that in *Xanthomonas phaseoli*, which catabolize glucose via the Entner-Doudoroff and pentose phosphate pathways and the tricarboxylic acid cycle (2, 21), a recycling is thermodynamically likely (2). Also in this species the CO yield from C6 exceeds that from C3 during the early phase of the experiment (21).

It does not seem likely that acetate formed from triose via pyruvate (C3 and C6 of glucose) should behave differently from acetate formed from the upper half of the glucose

TABLE 1

Species and strain	Labelled carbon	μmoles substrate	Phosphate-based medium CO_2	Cells	Medium	Total
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	2		66	7	21	94
	3		56	9	33	98
	4		54	12	30	96
	6		39	14	41	94
N subflava ATCC 11076	1	4	78	4	15	97
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	4		48	16	40	104
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	2		33	6	60	99
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ntinued)

NaCl/Tris-based medium					KCl/Tris-based medium					Heart Infusion Broth				
µmoles substrate	CO	Cells	Medium	Total	µmoles substrate	CO_2	Cells	Medium	Total	µmoles substrate	CO	Cells	Medium	Total
1.5	95	0	4	99	0.5	98	2	3	103	15	96	2	3	101
	76	1	15	92		77	9	14	100		56	20	17	93
	70	2	25	97		68	13	24	105		44	28	29	101
	79	3	12	94		54	20	24	98		44	24	12	80
	11	4	88	103		51	19	26	96		39	30	24	93
3	95	0	3	98	2	94	3	7	104	20	93	3	5	101
	53	1	42	98		71	10	23	104		60	16	19	95
	38	1	61	100		61	9	33	103		49	25	31	105
	88	1	13	102		79	17	19	115		50	19	16	85
	10	1	89	100		61	11	27	99		48	25	25	98
1	73	0	26	99	1	66	0	40	106					
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	32	7	56	95		25	17	59	101					
	3	8	97	108		8	17	76	101					

at the cessation of the CO evolution, usually after 5 hours using Heart Infusion Broth, and after

the pool of amino acids and other cellular components which are catabolized as the need for energy rises when the glucose has been consumed (20)

According to the present results, the tricarboxylic acid cycle seems to be as active in *N meningitidis* and *N gonorrhoeae* in which *in vitro* malate dehydrogenase activity has not been found (9 11) as in the other saccharolytic *Neisseria* species. The only exception is *N gonorrhoeae* 1 a in which the oxidation of acetate proceeds very slowly and incompletely possibly because of inefficient synthesis of citrate (6)

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It does not seem likely that acetate formed from triose via pyruvate (C3 and C6 of glucose) should behave differently from acetate formed from the upper half of the glucose

molecule (C2 and C3) The low CO_2 yield from C6 in KCl/Tris- (*N meningitidis* and *N gonorrhoeae*) and NaCl/Tris-based media (other saccharolytic *Neisseria*) is thus somewhat unexpected. The form of the C6 curve suggests that most of the CO_2 from C6 appears because of recycling of triose only a small fraction, if any can have arisen as a result of acetate oxidation. In these media, therefore, triose cannot be readily converted to pyruvate. Instead, the triose may be metabolized by other reactions, C4 being split off as CO_2, and the rest being excreted into the medium, possibly as a C_2 fragment. The "true neisserias" with the exception of *N elongata* are incapable of oxidizing acetate in minimal media (7) and may hence be unable to metabolize certain C_2 fragments under these conditions.

The overall metabolism of glucose in these media seems to be changed the catabolism is slower than in the other media all glucose is used for energy production, the organisms being unable to incorporate label into cellular components and the pathway from triose to pyruvate is partially blocked. These effects are associated with the mutual absence of phosphate and sodium ions in *N meningitidis* and *N gonorrhoeae* and of phosphate and potassium ions in the other saccharolytic *Neisseria*. Although only one strain from each of the species *N meningitidis* and *N gonorrhoeae* are fully examined (*N gonorrhoeae* 1 a deviates because of its very low oxidation of acetate) these findings indicate a difference in the regulation of glucose metabolism in pathogenic and non-pathogenic *Neisseria* species.

In *N meningitidis* the initial rate of oxygen consumption, using glucose as substrate, was not affected by phosphate, but the total oxygen uptake was reduced, probably because of diminished oxidation of acetate (14) The present results show a reduced CO_2 production from pyruvate carboxyl in phosphate-based medium as well as a depression of tricarboxylic acid cycle activity These effects of phosphate are more pronounced in *N meningitidis* and *N gonorrhoeae* than in the non-pathogenic *Neisseria*. It is known from radiorespirometric studies using glutamate as substrate (6) that phosphate depresses tricarboxylic acid cycle activity but not as much as when glucose is the substrate, and to the same extent in pathogenic and non-pathogenic *Neisseria*. Phosphate increases the CO_2 yield from C2 and C3 of pyruvate (7) This may be due to influence upon the synthesis of C_4 compounds from pyruvate rather than to a stimulation of tricarboxylic acid cycle activity A partial inhibition of phosphoenolpyruvate carboxylase by phosphate (16) seems to be a probable cause of the diminished oxidation of acetate.

The near identical curves of CO production from C4 and C6 in *N perflava* ATCC 10555 could be the result of an extensive recycling of triose. A randomization of C4 and C6 at the triose level, possibly in dihydroxyacetone phosphate, cannot be excluded. This might also lead to identical curves for C4 and C6. An explanation of the preferential oxidation of C3 over C2 in minimal media has not been found. Using pyruvate as substrate, the differential rate of CO_2 evolution is C1 $\gg$ C2 > C3 and, using acetate, it is C1 > C2 (7) which means that these substrates are oxidized in the usual way via the tricarboxylic acid cycle. The data found, using glucose as substrate, may thus indicate an aberration above the pyruvate level.

^{14}CO yield from glucose has been used to calculate concurrent pathways of glucose catabolism in microorganisms (19) To derive equations for this calculation it was assumed, among other things, that the C_3 units formed from glucose were equivalent to each other with respect to further metabolic reactions, that no significant recombination of triose to form hexose took place, and that there was no significant randomization of the hexose skeleton via the transketolase or transaldolase reactions (18, 19) These assumptions are not fulfilled in the present system. Owing to the recycling of triose the CO_2 yield from C1 and C4 will not directly reflect the participation of the Entner Doudoroff pathway in glucose catabolism. The possibility of triose

being shunted through reactions other than those leading to pyruvate, further complicates the picture. Moreover *Morse et al* (17) have mentioned the possibility that pentose also may be formed from triose and fructose phosphates in *N. gonorrhoeae*. Accordingly the equations of *Wang & Krackov* (19) cannot be used for an exact calculation of the participation of the Entner-Doudoroff and pentose phosphate pathways in these microbes. However without taking into account the extent of recycling of triose, it may roughly be estimated that about [illegible] of the glucose is catabolized via the pentose phosphate pathway and the rest, [illegible], via the Entner Doudoroff pathway dependent on the medium used. In phosphate based medium, more of the substrate is catabolized via the pentose phosphate pathway. The Entner Doudoroff pathway seems to be used to a greater extent in *N. meningitidis* and *N. gonorrhoeae* than in the non-pathogenic species. These data are in accordance with those obtained for *N. gonorrhoeae* (17) and, using a different method, for *N. meningitidis* (13).

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REFERENCES

1. *Gorini, L. & Gundersen, W.:* Induction by arginine of enzymes of arginine biosynthesis in *Escherichia coli* B. Proc. nat. Acad. Sci. *47* 961-971 1961.
2. *Hochster, R. M. & Katznelson, H.:* On the mechanism of glucose-6-phosphate oxidation in cell-free extracts of *Xanthomonas phaseoli* (XP8). Can. J. Biochem. Physiol. *36* 669-689 1958.
3. *Holten, E.:* Glutamate dehydrogenases in genus *Neisseria*. Acta path. microbiol. scand. Sect. B, *81* 49-58, 1973.
4. *Holten, E.:* Glucokinase and glucose 6-phosphate dehydrogenase in *Neisseria*. Acta path. microbiol. scand. Sect. B, *82* 201-206 1974.
5. *Holten, E.:* 6-phosphogluconate dehydrogenase and enzymes of the Entner-Doudoroff pathway in *Neisseria*. Acta path. microbiol. scand. Sect. B, *82:* 207-213 1974.
6. *Holten, E.:* Radiorespirometric studies in genus *Neisseria*. 2. The catabolism of glutamate and fumarate. (to be submitted)
7. *Holten, E.:* Radiorespirometric studies in genus *Neisseria*. 3. The catabolism of pyruvate and acetate. (to be submitted)
8. *Holten, E. & Jyssum, K.:* Glutamate dehydrogenases in *Neisseria meningitidis*. Acta path. microbiol. scand. Sect. B, *81* 43-48, 1973.
9. *Holten, E. & Jyssum, K.:* Activities of some enzymes concerning pyruvate metabolism in *Neisseria*. Acta path. microbiol. scand. Sect. B, *82* 843-848, 1974.
10. *Jyssum, K.:* Assimilation of nitrogen in meningococci grown with the ammonium ion as sole nitrogen source. Acta path. microbiol. scand. *46* 320-332, 1959.
11. *Jyssum, K.:* Intermediate reactions of the tricarboxylic acid cycle in meningococci. Acta path. microbiol. scand. *48* 121-132, 1960.
12. *Jyssum, K.:* Dissimilation of C^{14} labelled glucose by *Neisseria meningitidis*. 1. The formation of CO_2 and acetate from glucose carbon. Acta path. microbiol. scand. *55* 319-324 1962.
13. *Jyssum, K.:* Dissimilation of C^{14} labelled glucose by *Neisseria meningitidis*. 2. The incorporation of 1-C^{14} and 6-C^{14} into cellular components in short-time experiments. Acta path. microbiol. scand. *55* 325-334 1962.
14. *Jyssum, K., Borchgrevink, B. & Jyssum, S.:* Glucose catabolism in *Neisseria meningitidis*. 1. Glucose oxidation and intermediate reactions of the Embden-Meyerhof pathway. Acta path. microbiol. scand. *53:* 71-83 1961.
15. *Jyssum, K. & Joner, P. E.:* Dissimilation of C^{14} labelled glucose by *Neisseria meningitidis*. 3. The incorporation of 1-C^{14} and 6-C^{14} into pyruvate. Acta path. microbiol. scand. *55:* 335-341 1962.
16. *Jyssum, K. & Jyssum, S.:* Phosphoenolpyruvate carboxylase activity in extracts from *Neisseria meningitidis*. Acta path. microbiol. scand. *54* 412-424 1962.
17. *Morse, S. A., Stein, S. & Hines, J.:* Glucose metabolism in *Neisseria gonorrhoeae*. J. Bact. *120* 702-714 1974.
18. *Wang, C. H.:* Radiorespirometric methods. In Norris, J. R. & Ribbons, D. W. (Eds.): Methods in microbiology vol. 6 B. Academic Press, London and New York 1972 p. 185-230.
19. *Wang, C. H. & Krackov, J. K.:* The catabolic fate of glucose in *Bacillus subtilis*. J. biol. Chem. 237 3614-3622 1962.
20. *Wang, C. H., Stern, I., Gilmour, C. M., Klungsøyr, S., Reed, D. J., Bialy, J. J., Chri-*

stenson B E. & Cheldelin, V H.: Comparative study of glucose catabolism by the radiorespirometric method. J Bact. *76* 207–216, 1958.

21 *Zagallo A C. & Wang, C. H* Comparative glucose catabolism of *Xanthomonas* species. J. Bact. *93* 970–975, 1967

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APPLICATION OF THE PASSIVE HAEMOLYSIS TEST FOR THE DETERMINATION OF RUBELLA VIRUS ANTIBODIES

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Skaug, K., Ørstavik, I. & Ulstrup, J. C. Application of the passive haemolysis test for the determination of rubella virus antibodies. Acta path. microbiol. scand. Sect. B, 83: 367–372, 1975.

A passive haemolysis test for the determination of antibodies against rubella virus haemagglutinin is presented. According to this method, the principle of radial immunodiffusion technique is applied. Rubella haemagglutinin-coated chick erythrocytes in the agarose gel were lysed by the diffusing positive sera in the presence of complement at 37° C. The passive haemolysis test was compared with the conventional haemagglutination inhibition method, and the diameter of the haemolysis zones was shown to be a direct measure of the quantity of antibody added to the well. A plot of the log (HI titre) against the zone diameter gives a straight line. There is no need to remove nonspecific haemagglutination inhibitors. However, all serum samples must be inactivated at 56° C for 30 minutes before testing. Tests of 200 serum samples from healthy women showed a good correlation between the haemagglutination inhibition titre and the antibody titre determined by the passive haemolysis technique. Twenty-one samples with a haemagglutination inhibition titre less than 10 were also negative in the passive haemolysis test. With the exception of one, all sera with a positive haemagglutination inhibition titre showed a positive haemolysis reaction. The method was found to be as specific and as sensitive as the haemagglutination inhibition test. In addition, the technique is rapid and simple for quantitative studies of antibodies against rubella virus.

Key words: Rubella virus antibodies; passive haemolysis test; haemagglutination inhibition test.

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A number of serological tests for rubella virus antibodies have been developed (7). At present, the haemagglutination inhibition (HI) test is the one most commonly used. Following a rubella virus infection, the HI antibodies usually persist for years (3).

The HI test is considered a specific and sensitive test and, as compared with the other tests available, it is simple to perform. However, before testing for rubella virus HI antibodies, all sera must be pretreated in order to remove nonspecific inhibitors. This procedure has to be standardized and controlled and it is rather time consuming. A rapid and simple assay for determination of the HI antibodies is therefore of interest.

In the present report we describe a passive haemolysis (PH) test for rubella virus haemagglutinin antibodies, in which it is not neces-

sary to pretreat the sera. A similar test for influenza virus haemagglutinin antibodies has recently been developed by *Schild et al* (5) It is based on the principle that erythrocytes which are coated with haemagglutinating antigens will be lysed by haemagglutinin antibodies in the presence of complement.

MATERIALS AND METHODS

Red blood cell suspension Chick erythrocyte suspensions were prepared from pooled blood collected aseptically in Alsever's solution from unfed 1-day-old chicks. The erythrocytes were washed 3 times in dextrose-gelatin-veronal buffer (2) centrifuged at 1500 rev/min for 5 minutes and diluted to a 10 per cent stock suspension in veronal buffer and kept at 4 C.

Adult chick red blood cells (RBC) were prepared in the same way and stored as a 50 per cent suspension.

Rubella virus haemagglutinin The rubella virus haemagglutinin (HA) was obtained from Behringwerke AG (batch no. 192B) One bottle of the lyophilized HA was dissolved in 1 ml veronal buffer (containing 8.5 g NaCl, 0.575 g 5,5-diethylbarbituric acid, 0.2 g Na-5,5-diethylbarbiturate, 0 168 g $MgCl_2$ $6H_2O$ and 0.028 g $CaCl_2$ per litre) pH = 7.2. When titrated in the microtitre system (2) the antigen showed a titre 256, corresponding to about 10 000 HA units/ml.

Agarose A-37 (Indubiose, L Industrie Biologique Francaise) was dissolved in veronal buffer pH = 7.2 to a final concentration of 1.5 per cent (w/v) (stock solution)

Control sera One negative control serum (serum 0) and two positive control sera, one strongly positive (serum 640) and one weakly positive (serum 40) with a HI titre 640 and 40, respectively were used both in the HI test and in the PH test for development of the latter Serum 640 to be used in the PH test was diluted in serum 0.

Haemagglutination inhibition test The HI test was carried out according to the method described by *Halonen et al.* (2) the sera being pretreated with kaolin and using the microtitre system and the same antigen as in the PH test. The initial dilution of a serum sample in the test was 1 10.

Guinea pig complement solution 1.5 ml guinea pig serum was absorbed at 4 C for 30 minutes with 0.4 ml freshly washed and packed adult chick erythrocytes. After centrifugation the supernatant was diluted twofold with the veronal buffer The diluted complement solution was kept on ice bath until used in the PH test.

Sensitized erythrocytes One ml of the freshly washed chick erythrocytes was mixed with 1 ml of the rubella virus haemagglutinin. The suspension was kept at 4 C for 10 minutes to allow HA adsorption. Then the erythrocytes were centrifuged and resuspended in 2 ml veronal buffer. This stock suspension containing 5 per cent sensitized erythrocytes was kept at 4 C until used.

Passive haemolysis diffusion plates 3 ml of the agarose stock solution were melted and then cooled to about 45 C before 0.3 ml of the sensitized erythrocytes was added. Immediately afterwards, the mixture was carefully shaken and poured into an immunodiffusion plate (Hyland Inc.)

After gelation, 2.5 mm wells were punched out. The plates were kept at 4 C and were used the same day

The passive haemolysis test: The samples were inactivated at 56° C for 30 minutes before 3 µl volumes were added to the wells. The immunoplates were placed at room temperature to allow the sample to disappear from the well which then was filled with melted agarose. The diffusion plate was flooded with 0.2 ml of the guinea pig complement solution. After incubation at 4 C for 18 hours, the haemolysis developed within 1 hour at 37 C. Two perpendicular diameters of the haemolysis zone were measured, A standard curve was made up by comparing the zone diameters of different dilutions of serum 640 with the corresponding HI titres.

As a control for nonspecific lysis, the samples were tested on immunoplates with normal chick erythrocytes.

Serum samples Sera from 200 healthy [illegible] aged 20–30 years were tested.

RESULTS

Optimal erythrocyte and antigen concentration To obtain the most distinct haemolysis zones, experiments with varying concentrations of RBC sensitized with different concentrations of the rubella virus HA were carried out. The results are presented in Table 1 A final concentration of 0.5 per cent RBC was chosen (1 ml 10 per cent RBC sensitized with 1 ml HA) This concentration gave an easily readable haemolysis zone. The sharpness of the zone limit could be increased somewhat by increasing the antigen concentration. However within an antigen concentration range from 2,500 to 20,000 HA units, the zone diameter did not change significantly At higher concentrations of RBC, many sera showed an incomplete haemolysis around the well which was absent under the chosen conditions.

TABLE 1. *Distinctness of the Haemolysis Zones in the PH Plates After a Diffusion Period of 18 Hours with Varying Concentrations of Erythrocytes Sensitized with Different Concentrations of the HA. The Experiments Were Performed with Serum 640*

HA units/ml	Distinctness of the haemolysis zones with different final concentrations of the RBC, per cent			
	2	1	0.5	0.25
20,000	+ +	+ + +	+ + +	+
10,000	+	+ +	+ + +	+
2,500	+	+ +	+	−

+, + +, + + + Degree of haemolysis.

Development of the haemolysis zone. Preliminary experiments with native sera and with sera heated at 56° C for 30 minutes showed that most of the nonheated sera gave a haemolysis zone also in the control plate. When the sera were preheated, no haemolysis zone was observed in the control plate. All the tests were therefore carried out with preheated serum samples. To secure that the complement was not a limiting factor the diffusion plates were flooded with varying volumes of guinea pig serum complement. In order to develop the haemolysis zones within one hour at 37° C, at least 0.2 ml of the complement solution was necessary. This amount was also necessary to cover the plates completely. Development of the haemolysis would be more rapid if complement was added before rather than after incubation at 4° C.

Diffusion time. The influence of different periods of diffusion of the control serum appears from Fig. 1. Haemolysis zones were difficult to measure after 43 hours at 4° C owing to the spontaneous lysis of the RBC. Up to this time, as shown in Fig. 1, a linear correlation between the zone diameters and the HI titres was observed in a semilogarithmic plot. For practical reasons, the diameter of the haemolysis zones were measured after 18 hours of diffusion.

Sensitivity and reproducibility of the passive haemolysis test. By extrapolating the curves in the semilogarithmic plot they were found to intersect at the same HI antibody titre of about 1, which corresponded to the well diameter (Fig. 1). By testing dilutions of serum 640, 2 HI antibody units were easily readable.

By comparing the diameters of 24 samples of the control serum, diluted to contain 160 HI titre units, on different plates and in different runs, there was very little variation

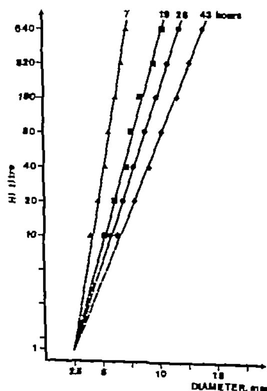


Fig. 1. The correlation between the diameter of the haemolysis zones and the HI titres obtained by several dilutions of serum 640, after different periods of diffusion.

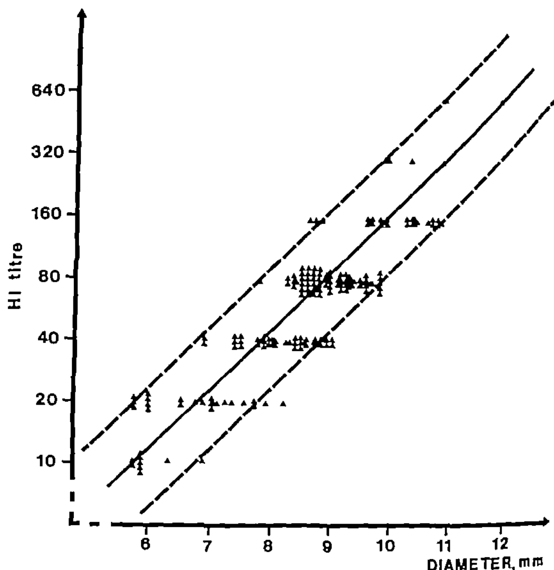


Fig. 2 Correlation between 178 positive results obtained by the HI and the PH test (single determinations) The solid line indicates the same antibody titre in both test, and the dotted lines indicate limits of ± one titre-step.

in zone diameter The standard deviation of the mean was approximately 3 per cent.

Test results To evaluate the method in routine work, 200 sera from healthy women were examined by the HI method and by the PH test. Only single determinations were performed. By comparing the zone diameters with the standard curve, the HI antibody titre of the sera were determined. The correlation between the HI test and the PH test of 178 successive positive samples is shown in Fig. 2. There was good correlation between the HI titre and the antibody titre determined by the PH technique. Twenty-one samples with HI titres less than 10 were also negative in the PH test. With the exception of one, all sera with a positive HI titre showed a positive PH activity The one serum which disagreed showed a very high nonspecific HI titre and is subjected to further investigations.

In Fig. 3 are presented two photographs of the PH test, photographed after 18 hours of diffusion, of serum 0 and several dilutions

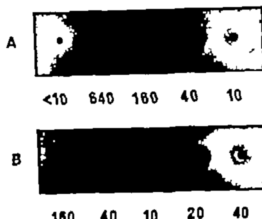


Fig. 3. The haemolysis zones in the PH plates photographed after 18 hours. A: Serum 0 and different dilutions of serum 640 for the construction of a standard curve. B: Results obtained by different serum samples. The HI titres are recorded below the corresponding serum well.

of serum 640, respectively, and of some of the serum samples.

DISCUSSION

As regards the PH method, the principles of the radial immunodiffusion technique are applied in the reversed form, i.e. the antibodies are permitted to diffuse in an antigen containing gel. *Leerman et al.* (6) found no disparity between the results of the reversed and the original immunodiffusion technique.

In contrast to the reversed radial immunodiffusion technique where the antigen molecules are distributed in the gel, the antigen used for the PH method is fixed to erythrocytes which are interspersed in the gel. We found that the concentration of the HA antigen on the surface of the erythrocytes, within certain limits, did not significantly influence the size of the haemolysis zones. In the case of influenza virus HA antigen, similar results have been reported by *Schild et al.* (5) who used the PH method. In our experiments, the diameters varied with the content of rubella virus HI antibody in the test samples, and with the diffusion period. As regards diffusion periods between 7 to 43 hours, linear correlations between the diameter of the haemolysis zones and the HI titres in a semi-logarithmic plot were obtained. Accordingly the PH method is readily standardized.

Different techniques have been used to describe the results of the radial immunodiffusion technique (1–4). In our experiments, the haemolysis zones were measured while the areas were still expanding. A plot of the zone diameter against the log (HI titre) could best be fitted with straight lines, corresponding with results previously obtained by *Fahey & McKelvey* (1).

The present method involves several practical advantages. Firstly, the serum can be tested undiluted. Furthermore, in contrast to the HI test, it is not necessary to remove the nonspecific inhibitors of haemagglutination. The pretreatment can be limited to heating the serum at 56° C for 30 minutes in order to inactivate factors lysing normal chick erythrocytes. As a third advantage, the PH test was found to be slightly more sensitive than the HI test, as it was possible to measure a haemolysis zone corresponding to an antibody concentration of 2 HI antibody units.

In our studies, the PH method appeared to be as specific for the demonstration of antibody against rubella virus HA as the conventional HI method. The results of 178 positive and 21 negative sera, out of 200 tested, agreed with those obtained by the HI test. One serum was positive in the HI test and negative in the PH test. This serum showed a very high nonspecific HI titre and is subjected to further investigations. There was also good quantitative agreement between the two methods. In the HI test, as used here, the lowest titre measurable is 10. In the PH test, haemolysis zones corresponding to antibody titres less than 10 were not observed among the 200 sera tested. Serum 640, diluted to present an antibody titre 2, still gave a measurable haemolysis zone. To which extent small haemolysis zones in single sera also will be specific can only be determined by further experience.

We found the PH method to be a simple and reproducible test for the quantitation of rubella virus haemagglutinin antibodies and

well suited for testing large numbers of serum samples.

The authors would like to thank Miss *Turid Vollen* for excellent technical assistance.

REFERENCES

1. *Fahey J L & McKelvey E M* Quantitative determination of serum immunoglobulins in antibody agar plates. J Immunol. *94* 84–90, 1965
2. *Halonen P E, Ryan J M & Stewart J A* Rubella hemagglutinin prepared with alkaline extraction of virus grown in suspension culture of BHK 21 cells. Proc. Soc. Exp. Biol. Med. (N Y) *125* 162–167 1967
3. *Horstmann, D M & Liebhaber H* Rubella In Paul J R. & White, C. (Ed.): Serological epidemiology Academic press, New York and London, 1973 p. 83–97
4. *Mancini, G., Carbonara A O & Heremans, J F*: Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry *2* 235–254 1965
5. *Schild G C Oxford J S & Virelizier J L*: Immunity to Influenza. 1975 in press.
6. *Vaerman J P Lebacq-Verheyden A M Scolari L & Heremans J F* Further studies on single radial immunodiffusion—II The reversed system Diffusion of antibodies in antigen-containing gels. Immunochemistry *6* 287–293 1969
7. *Vesikari, T.* Immune response in rubella infection. Scand. J Infect. Dis., suppl. 4 1972.

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CHARACTERIZATION OF *MICROCOCCACEAE* FROM THE URINARY TRACT

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Two hundred and seven urinary strains of staphylococci and micrococci were classified biochemically according to *Baird-Parker* (1963) and by means of a simplified schema. One hundred and thirteen strains belonged to *Staphylococcus aureus* *S epidermidis* or *S saprophyticus* (64 strains) according to the simplified schema, respectively to Baird-Parker's subgroups SI, SII or M3 *S saprophyticus* was isolated from young, female out-patients, was relatively resistant to novobiocin and contained poly A+C (β-N-acetylglucosaminyl ribitol teichoic acid and β-N-acetylglucosaminyl glycerol teichoic acid) *S aureus* and *S epidermidis* were isolated from older male in-patients, were sensitive to novobiocin and contained poly A (N-acetylglucosaminyl ribitol teichoic acid) respectively poly B (glucosyl glycerol teichoic acid) Ninety-four strains belonging to other *Staphylococcus* or *Micrococcus* subgroups could not be classified by the simplified schema. With few exceptions, these strains were sensitive to novobiocin and either gave a precipitin reaction corresponding to poly C or were non-typable with the teichoic acid reference systems used. The simplified schema is recommended for the classification of coagulase-negative strains of *Micrococcaceae*

Key words *Micrococcaceae* urinary tract; characterization.

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Members of the family *Micrococcaceae* sometimes cause infections of the urinary tract. The separation of coagulase-negative staphylococci from micrococci is a problem in the bacteriological laboratory The standard glucose fermentation test (24) is not satisfactory as a group of staphylococci often found in urinary tract infection (11 13 14 20 21) attack glucose very slowly anaerobically and, in contrast to other staphylococci, give a negative reaction. These cocci were consequently regarded as micrococci of Baird Parker's subgroup 3 (13 14 21) They have later been shown to have a DNA base composition (8, 16) a type of peptidoglycan (22 23) and wall teichoic acid (5 19) corresponding to genus *Staphylococcus* and are now being classified as *S saprophyticus* (3) They are resistant to novobiocin (13 14 15 20) and contain both ribitol and glycerol teichoic acid in their walls (5 6 11 19)

There is a diagnostic need for a better knowledge of the clinical significance and taxonomic characteristics of the various *Micrococcaceae* species and subgroups isolated from the urine, the properties of *S saprophyticus* being of particular interest. In this report, two series of urinary strains primarily diagnosed in the routine as staphylococci or

micrococci have been submitted to more detailed investigation.

MATERIALS AND METHODS

Strains

Strains isolated in the diagnostic laboratory from urinary specimens and diagnosed as *S aureus*, *S epidermidis* or *Micrococcus* were during two 6-month periods, October–April 1971/72 and October–April 1972/73, preserved on nutrient agar for further examination. The majority of the strains were isolated from midstream specimens. The urines from out-patients were usually added 1.8 per cent boric acid, or in some cases were sent on a dip-slide. In the laboratory every urine was examined in a Gram-preparation and inoculated with a calibrated loop on bromthymolblue lactose agar and blood agar plates.

In the first period 123 urinary specimens were found to contain staphylococci or micrococci in significant amounts. Thirty-three of these strains, in addition to 4 strains from non-significant bacteriuria, all chosen at random, were included in the material. In the second period 160 specimens were found to contain staphylococci or micrococci in significant amounts. 152 of these strains, in addition to 25 strains from non-significant bacteriuria, being included in the material. Six strains from specimens with significant bacteriuria and one strain from non-significant bacteriuria were excluded after the examination had shown that they were identical to a previously isolated strain from the same patient. The material finally evaluated biochemically for novobiocin sensitivity and serologically for type of teichoic acid, consisted of 207 strains.

Patients

Age, sex, in- or out-patients and pure or mixed culture were correlated with the classification of the 178 independent strains isolated from specimens with significant bacteriuria. Criteria for significant bacteriuria were $>10^5$ bacteria/ml urine, semiconfluent or confluent growth in dip-slide culture (Uricult) or bacteriuria on bladder puncture. The bacteriological diagnosis was confirmed through a second specimen in 6 cases only.

Biochemical Tests

The strains were classified into staphylococcal and micrococcal subgroups according to the schema of *Baird-Parker* (1). The standard test for examination of glucose fermentation (24) was used. Acid production aerobically from glucose was examined both in broth and in agar culture, and from mannitol, lactose and arabinose in agar culture only. In addition a simplified set of biochemical tests was used for the classification of the strains. This set consisted of four tests: coagulation of rabbit plasma, acid production aerobically from mannitol, production of phosphatase and examination on arginine dihydrolase activity (17). According to *Kocur* (7) these tests give the following reaction patterns in the three *Staphylococcus* species:

	S aureus	*S saprophyticus*	*S epidermidis*
Coagulase	+	—	—
Acid aerobically from mannitol	+	+	—
Phosphatase	+	—	+
Arginine dihydrolase	+	—	+

In the following, S or M together with the number of the subgroup indicates classification by means of Baird-Parker's schema (1), while *S. aureus*, *S saprophyticus* and *S epidermidis* mean classification by the simplified biochemical set. Strains typable with both schemas are reported by a combination of the two designations, e.g. *S. aureus*/SI.

Novobiocin Sensitivity

Novobiocin Neo-Sensitabs (Rosco) were used on Mueller Hinton culture medium (Difco) without blood. According to the manufacturer inhibition zones ≥28 mm were interpreted as sensitivity (MIC ≤1.5 µg/ml), 23–27 mm as moderate sensitivity (MIC >1.5 µg/ml≤6 µg/ml), 15–22 mm as relative resistance (MIC>6 µg/ml≤50 µg/ml) and ≤14 mm as resistance (MIC >50 µg/ml).

Agar Precipitation

The type of wall teichoic acid was determined by double diffusion in agar gel using reference systems consisting of rabbit immune sera and polysaccharide (teichoic acid) preparations (18). The following references were included:

Poly Aα (*S aureus* α-N-acetylglucosaminyl ribitol teichoic acid)
Poly Aβ (*S aureus* β-N-acetylglucosaminyl ribitol teichoic acid)
Poly Bα (*S epidermidis* α-glucosyl glycerol teichoic acid)
Poly Bβ (*S epidermidis* β-glucosyl glycerol teichoic acid)
Poly AβC (*S saprophyticus* β-N-acetylglucosaminyl ribitol teichoic acid and β-N-acetylglucosaminyl glycerol teichoic acid (poly C) (5, 6)

TABLE 1 *Novobiocin Sensitivity and Content of Precipitinogens in 113 Strains Which Could be Classified by Both Biochemical Schemas*

Biochemical classification S	B-P		Novobiocin sensitivity			Precipitinogens						
			1	2	3	Aβ/Aαβ Prot. A	Aα/Aβ	Ba	AβC	C	Others	NT
S aur./SI		13	12	0	1	9	4	0	0	0	0	0
S hyi./SII		32	32	0	0	0	0	25	1	1	2	3
S sapro./SI3		64	0	18	46	0	1	0	61	0	2	0
S sapro./SI3		4	2	0	2	0	0	1	1	1	1	0
		113	46	18	49	9	5	26	63	2	5	3

S = simplified schema, B-P = Baird-Parker' schema (1)
Novobiocin Neo-Sensitabs (Rosco)
1 = sensitive (MIC ≤1.5 μg/ml) 2 = moderately sensitive (MIC >1.5 μg/ml ≤6 μg/ml) 3 = relatively resistant (MIC >6 μg/ml ≤50 μg/ml)
NT = not typable.

TABLE 2 *Novobiocin Sensitivity and Content of Precipitinogens in 92 Strains Which Could be Classified by Baird-Parker's Scheme (1) Only*

Biochemical classification		Novobiocin sensitivity			Precipitinogens						
		1	2	3	Aβ/Aαβ Prot. A	Aα/Aβ	Bα	AβC	C	Others	NT
SII	2	2	0	0	0	0	0	0	1	0	1
SIII	1	0	0	1	0	1	0	0	0	0	0
SIV	9	9	0	0	0	0	1	0	3	0	3
SV	13	13	0	0	0	0	7	0	1	1	4
SVI	31	31	0	0	0	2	1	0	14	2	12
M1	3	3	0	0	0	0	0	0	1	1	1
M.	18	13	1	4	0	0	0	3	10	1	2
M3	1	1	0	0	0	0	0	0	0	0	1
M5	8	7	1	0	0	1	0	0	3	0	4
M6	3	2	1	0	0	1	0	0	1	0	1
M7	3	2	1	0	0	0	0	0	1	0	2
Unclassified	2	2	0	0	0	0	0	0	2	0	0
	94	83	4	5	0	5	9	5	37	5	33

See footnotes in Table 1

RESULTS

Biochemical Classification

Two hundred and five strains could be classified by one or both of the two biochemical schemas used, two strains could not be classified (Table 1 Table 2) One hundred and thirteen strains could be classified by both schemas (Table 1) A complete correlation was found between *S aureus* and S subgroup I All *S epidermidis* strains fell into S subgroup II, whereas two SII strains could not be classified by the simplified set. All typical M subgroup 3 strains corresponded to *S saprophyticus*. Four biochemically typical *S saprophyticus* strains were classified by Baird-Parker's schema in M subgroup 5 two of them giving atypical reactions (Table 1)

The 92 strains which could not be classified by the simplified set were distributed on nearly all of Baird Parker's S and M subgroups (Table 2) Five subgroups contained eight strains or more, *viz.* SIV SV SVI M2 and M5 the high incidence of SVI strains being the most interesting finding

Novobiocin Sensitivity

One biochemical group, *i.e.* *S saprophyticus*/M3 was clearly different from all other groups with regard to novobiocin sensitivity (Table 1) Of 64 strains belonging to this group 46 were relatively resistant and 18 were moderately sensitive having zones close to relative resistance. In contrast all of the 32 *S epidermidis*/SII strains and all except one of the 13 *S aureus*/SI strains were sensitive to novobiocin. Of the 7 other relatively resistant strains, two were *S saprophyticus*/M5 strains and four were M2 strains (Table 1 Table 2)

Agar Precipitation

All the 13 *S aureus*/SI strains contained poly A (Table 1) In 12 strains the amino sugar determinant was β linked, in one strain α-linked and in five strains both anomers were present. Nine strains also contained protein A. Of the 32 *S epidermidis*/SII strains 25 contained poly B. In all the poly B strains the glucose was α linked. Sixty-one of the 64 *S saprophyticus*/M3 strains contained poly AβC, one strain contained poly Aβ Of the four *S saprophyticus*/M5 strains one contained poly AβC.

Of the 9? strains which could be classified by Baird Parker's schema only the majority contained poly C (42 strains) or were non-typable (33 strains) (Table 2) Poly C was recognized most often in the subgroups SVI and M2. As a rule poly C was not found in combination with poly Aβ However of the 18 subgroup M2 strains 10 contained poly C alone and 5 contained poly AβC, 4 of the latter being relatively resistant to novobiocin. Seven out of 13 subgroup SV strains contained poly B.

Patients

The sex distribution presented in Table 3 shows one striking feature of 60 patients with significant amounts of *S saprophyticus*/M3 (poly AβC) in their urine 59 were women. The group "Other staphylococci" showed a slight dominance of women and "Other micrococci" a strong dominance. The distribution of the total material was 70 per cent women and 30 per cent men. The groups *S aureus*/SI and *S epidermidis*/SII show a predominance of men. *S saprophyticus*/M3 was isolated in approximately 50 per cent of the female cases and *S aureus*/SI or *S epidermidis*/SII in approximately 50 per cent of the male cases.

Of the 59 women with an *S saprophyticus*/M3 infection 55 (93 per cent) were out patients. This is significantly higher than the mean of the total material, which was 63 per cent for out-patients, the female mean being 78 per cent. In the groups "Other staphylococci" and "Other micrococci" the percentage female out patients is approximately the female mean, whereas women with *S aureus*/SI and *S epidermidis*/SII in their urine are definitely more often in-patients. Of the total of 54 men 43 per cent were out patients of those with *S aureus*/SI or *S epidermidis*/SII in their urine only 17 per cent were out-patients.

TABLE 3 *Evaluation of 178 Patients with Significant Amounts of Micrococcaceae in Their Urine*

	No. of patients		In-patients	Out patients	Age, mean	Pure culture	Mixed culture
S aur./SI	12	F 5 M 7	4 5	1 2	53 71	6	6
S epid./SII	28	F 12 M 16	6 14	6 2	41 63	23	5
S sapro./M3 (poly AβC)	60	F 59 M 1	4 0	55 1	28	49	11
Other staph.	52	F 29 M 23	9 9	20 14	32 60	36	16
Other microc.	26	F 19 M 7	4 3	15 4	36 53	17	9

F = female, M = male.

The mean age of women with *S saprophyticus*/M3 in their urine was 28 years. This is not very different from the mean age of the total female material, which was 31 years, or from those of the groups "Other staphylococci" or "Other micrococci" which was 32 and 36 years respectively The age of women with *S aureus*/SI or *S epidermidis*/SII bacteria in their urine was definitely higher The mean age of the total male material was 61 years, no great variations from this being found in the different groups of bacteria.

Mixed cultures were found in 47 cases (26 per cent) relatively often in the *S aureus*/SI group but also in the other groups of strains. Thirteen cultures contained other bacteria in insignificant amounts, 34 in significant amounts. Several bacteria were isolated, particularly members of the *Enterobacteriaceae* in addition to enterococci. In three cases significant amounts of two different strains of staphylococci were isolated.

The three most frequent subgroups of Table 2, *viz.* SV SVI and M2 were analyzed for the distribution of sex, age and in or out-patients. No special pattern was found in subgroups SV or SVI. Of the 11 cases with significant bacteriuria in subgroup M2, 3 strains contained poly AβC, were relatively resistant or moderately sensitive to novobiocin and were isolated from young women (age 20 21 26) They seem to be biochemically atypical *S saprophyticus* strains.

During the second 6-month period of the investigation a total of 3128 specimens with significant bacteriuria was diagnosed in the laboratory Seventy four per cent of the specimens were from women and 63 per cent were from out patients. Thus the sex and in- or out patient distribution of the *Staphylococcus*/*Micrococcus* material is not significantly different from that of the total urinary material. Samples containing staphylococci or micrococci constituted 5 per cent of the total material of significant bacteriuria.

Of the 28 strains of staphylococci or micrococci found in insignificant amounts eight were of subgroup SVI, seven of subgroup M2 and four were *S epidermidis*/SII strains. This might indicate that SVI and M2 strains often are contaminants, although all these strains were isolated in pure culture.

DISCUSSION

In this Department staphylococci or micrococci constitute about 5 per cent of the total number of cases with significant bacteriuria. The diagnostic laboratory should be able to classify coagulase-negative strains. As *S. saprophyticus* probably is the most usual of

these organisms in urinary tract infections (12, 21) a reliable diagnosis of this organism is of particular interest. Glucose fermentation is misleading due to the weak and slow anaerobic acid formation (8, 16) resulting in the registration of *S saprophyticus* strains as micrococci of subgroup 3. Also, this test takes at least five days, which is too much in routine work. The relative resistance of *S saprophyticus* to novobiocin has been advocated as a simple diagnostic test (2, 12). Resistance to novobiocin seems to occur only in the true *S saprophyticus*/M3 strains (19 present investigation) and not, as maintained, also in other Micrococcus subgroups (4 15). Nevertheless, the basing of classification on an antibiotic sensitivity test may be questionable.

The *S saprophyticus* strains classified by the simplified identification schema evaluated in the present investigation correlated very closely to *Micrococcus* subgroup 3 determined according to *Baird Parker* (1). The strains classified by both procedures as *S saprophyticus*/M3 were also very homogenous with regard to their type of teichoic acid, novobiocin sensitivity and clinical characteristics. Further the simplified schema correlated completely with Baird Parker's schema in the classification of *S aureus* (SI) and the true *S epidermidis* strains (SII). The tests of the simplified schema are easy and rapid to perform. They seem to separate at least urinary strains of the three *Staphylococcus* species reliably placing *S saprophyticus* in its correct genus. *Staphylococcus* strains not belonging to the three species, and strains of micrococci (except M3) cannot be classified by the simplified schema but probably are of less importance in human infections.

On investigation of a routine material, like the present, it is not possible to fulfil the requirement of at least two successive specimens of urine containing the same organism in significant amounts. This, in addition to a mixed flora in 26 per cent of the specimens, leaves a certain degree of doubt whether the isolated *Staphylococcus/Micrococcus* was, in every case, the causative organism. This may have affected the data presented in Table 3. However these data are in very good accordance with previous findings (12, 13 14). *S saprophyticus* usually being demonstrated in the urine of young, female out-patients and *S aureus* or *S epidermidis* in older male in-patients. The present material throws some doubt on the relationship between *S saprophyticus* infection and low age of the affected women (12, 13) as the mean age of this group was not very much lower than that of the total female material.

The serological identification of the type of wall teichoic acid correlated very well with the biochemical classification of *S aureus* *S epidermidis* and *S saprophyticus*. Of the 64 strains belonging to the latter species 61 strains contained poly AβC, this polysaccharide being demonstrated in only seven additional strains, five of which belonged to subgroup M2. Polysaccharide AβC is a mixture of β-N-acetylglucosaminyl ribitol teichoic acid and β N-acetylglucosaminyl glycerol teichoic acid (poly C) (10 19) the latter being characterized recently by *Johnsen et al.* (5 6). *S saprophyticus* has been stated to correspond to *Micrococcus* subgroups 1–4 (3 23).

The present material includes few strains belonging to subgroups M1 2 and 4 but out of 18 M2 strains only five contained poly AβC. Some biochemically atypical *S saprophyticus* urinary strains therefore seem to fall into *Micrococcus* subgroups other than M3 the majority of these subgroups containing strains which have not the criteria of *S saprophyticus* biochemically or with regard to novobiocin sensitivity or type of teichoic acid. It seems that the type of teichoic acid should be recognized as an important characteristic in the classification of these strains.

In all the 13 *S aureus* strains the characteristic poly A (N-acetylglucosaminyl ribitol teichoic acid) was demonstrated and in 25 out of the 32 *S epidermidis* strains the characteristic poly B (glucosyl glycerol teichoic acid). Poly B was demonstrated in 10 additional strains, seven of which belonged to

Staphylococcus subgroup V This subgroup has recently been included in S subgroup II (2) The present and other investigations (9) indicate that this is not a good solution According to the present results subgroup SV consists of biochemically atypical strains containing the teichoic acid of the true *S epidermidis* (SII) type, but also strains with no teichoic acid or other types in their walls.

Micrococci are said to have no wall teichoic acid (3) Of the 36 present strains belonging to *Micrococcus* subgroups other than *S saprophyticus*/M3 only 11 strains produced no line in our precipitation system. The significance of the poly C line, which was present in 16 of the *Micrococcus* strains, should be considered. It suggests the presence in these strains of a teichoic acid identical or similar to poly C, or some other cross-reacting polysaccharide material.

REFERENCES

1 *Baird-Parker A C:* A classification of micrococci and staphylococci based on physiological and biochemical tests. J gen. Microbiol. *30* 409–427 1963

2. *Baird-Parker A. C.* Classification and identification of staphylococci and their resistance to physical agents. In Cohen, J O (Ed.) The staphylococci. Wiley-Interscience, New York 1972, pp. 1–20.

3 *Baird-Parker A C* The basis for the present classification of staphylococci and micrococci. Ann. N Y Acad. Sci. *236* 7–13 1974

4 *Jeffries L.* Menaquinones in the classification of *Micrococcaceae* with observations on the application of lysozyme and novobiocin sensitivity tests. Int. J system. Bact. *19* 183–187 1969

5 *Johnsen G Skoge Endresen C Grov A & Oeding, P* Studies on polysaccharide C of *Staphylococcus epidermidis*. 1 Isolation and chemical characterization. Acta path. microbiol. scand. Sect. B, *83* 226–234 1975.

6. *Johnsen G. Skoge Grov A. & Oeding, P* Studies on polysaccharide C of *Staphylococcus epidermidis*. 2. Antigenic properties. Acta path. microbiol. scand. Sect. B, *83* 235–239 1975

7 *Kocur M.*, Personal communication.

8. *Kocur M., Bergan T & Mortensen, N.* DNA base composition of Gram-positive cocci. J gen. Microbiol. *69* 167–183 1971

9 *Krynski, S., Samet A & Becla E.:* Physiological and ecological differences between Baird Parker's *Staphylococcus* subgroup II and V XIth Conference on the taxonomy of bacteria, Brno, Czechoslovakia Sept. 1974 Abstracts.

10. *Losnegard N & Oeding, P* Immunochemical studies on polysaccharides from *Staphylococcus epidermidis*. 1 Isolation and chemical characterization. Acta path. microbiol. scand *58* 482–492, 1963.

11 *Losnegard N & Oeding, P.* Immunochemical studies on polysaccharides from *Staphylococcus epidermidis*. 2. Antigenic properties. Acta path. microbiol. scand. *58*. 493–500, 1963

12. *Maskell, R.* Importance of coagulase-negative staphylococci as pathogens in the urinary tract. Lancet *I* 1155–1158, 1974

13 *Mitchell R. G.* Staphylococci and urinary infection. Brit. Med. J. *I* 1127 1963.

14 *Mitchell R. G.* Classification of *Staphylococcus albus strains isolated from the urinary* tract. J clin. Path. *21* 93–96, 1968.

15 *Mitchell, R G & Baird-Parker A C.* Novobiocin resistance and the classification of staphylococci and micrococci. J appl. Bact. *30*: 251–254 1967

16. *Mortensen N & Kocur M* Correlation of DNA base composition and acid formation from glucose of staphylococci and micrococci. Acta path microbiol. scand. *69* 445-457 *1967*

17 *Møller V* Simplified tests for some amino acid decarboxylases and for the arginine dihydrolase system. Acta path. microbiol. scand. *36* 158–172, 1955

18. *Oeding, P* Wall teichoic acids in animal *Staphylococcus aureus* strains determined by precipitation. Acta path. microbiol. scand. Sect. B *81* 327–336, 1973.

19 *Oeding, P & Hasselgren I L.* Antigenic studies of genus *Micrococcus*. 2. Double diffusion in agar gel with particular emphasis on teichoic acids. Acta path. microbiol. scand. Sect. B, *80* 265–269 1972.

20. *Pereira, A Torres* Coagulase-negative strains of *Staphylococcus* possessing antigen 51 as agents f urinary infection. J clin. Path. *15* 252–253, 1962.

21 *Robert A P* *Micrococcaceae* from the urinary tract in pregnancy J clin. Path. *20* 631–63? 1967

22. *Schleifer K H & Kandler O* Amino acid sequence of the murein of *Planococcus* and other *Micrococcaceae* J Bact. *103*: 387–392, 1970.

23 *Schleifer K H & Kandler O.:* Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bact. Rev *36* 407-477 1972.

24. *Subcommittee.* Minutes of first meeting of subcommittee on taxonomy of staphylococci and micrococci. Int. Bull. Bact. Nomen. Tax. *15* 107–110, 1965.

25. *Subcommittee.* Minutes of third meeting of subcommittee on taxonomy of staphylococci and micrococci. Int. J. system. Bact. *21* 161–163 1971

COMMON ENTEROBACTERIAL ANTIGEN IN *YERSINIA ENTEROCOLITICA*

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Mæland, J. A. & Digranes, A. Common enterobacterial antigen in *Yersinia enterocolitica*. Acta path. microbiol. scand. Sect. B, 83: 382–386, 1975.

Production of the common enterobacterial antigen (CA) by strains of *Yersinia enterocolitica* (Y. e.) serotypes 3 and 9 (Winblad), by strains of 5 different Y. e. serogroups (Wauters) and various other bacteria was examined. Antibody against CA was raised by immunization of rabbits with *E. coli* O 14. Extract prepared from *S. typhimurium* was used for the sensitization of sheep erythrocytes with CA. Absorption and haemagglutination inhibition experiments showed that CA could be detected in heat extracts from all Y. e. strains examined, and in that from *Yersinia pseudotuberculosis*. CA was not detected in extracts from *Pasteurella multocida*, *Francisella tularensis*, *Brucella abortus*, *Acinetobacter calcoaceticus* or *Pseudomonas aeruginosa*. Anti-CA antibodies could not be demonstrated in serum from rabbits immunized with Y. e. bacteria, but were demonstrated in serum from rabbits immunized with a CA-containing fraction of the Y. e. extract. The possibility of participation of anti-CA antibody in indirect haemagglutination tests for detection of antibody to Y. e. O antigens is emphasized.

Key words: *Yersinia enterocolitica*; common enterobacterial antigen.

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Evidence has been provided that the common enterobacterial antigen (CA) is produced by all smooth strains and most rough strains of members of the *Enterobacteriaceae* (7, 18). The demonstration of this antigen in bacteria may be used for taxonomic purposes (8, 18). In 1972, *Le Minor et al.* (8) reported that CA could be detected in heat extract from *Yersinia enterocolitica*, *Yersinia pestis* and *Yersinia pseudotuberculosis*. The results are consistent with the present classification of *Yersinia* as a genus in the family *Enterobacteriaceae* (2).

Although most intestinal bacilli fail to engender production of anti-CA antibodies in rabbits (7, 17), antibody against CA can be elicited using *E. coli* O 14 (7). Erythrocytes sensitized with CA from *S. typhimurium* are commonly employed for the demonstration of anti-CA antibodies (1, 14, 16).

Extracts from *Y. enterocolitica* strains and from various other bacteria have been examined for the presence of CA, and antiserum against *Y. enterocolitica* strains has been tested for anti-CA antibodies.

MATERIALS AND METHODS

Bacterial Strains

Yersinia enterocolitica (Y. e.) serotypes 3 and 9 (Winblad) were received by courtesy of Professor *Winblad*, Malmö, Sweden. Y. e. serogroups 1, 2a, 3, 9, 13,7, 6,31, 6,30, 5 (Wauters), *Yersinia pseudotuberculosis* I, *Francisella tularensis* and

Brucella abortus were received from The National Institute of Public Health, Oslo, Norway *E. coli* O 14 and *Salmonella typhimurium* were obtained from The State Serum Institute, Copenhagen, Denmark. *Pasteurella multocida*, *Acinetobacter calcoaceticus* and *Pseudomonas aeruginosa* were isolated in our laboratory The bacteria were kept lyophilized.

Culture

All strains were cultivated on agar plates containing meat extract supplemented with peptone (1 per cent) and NaCl (0.5 per cent) except for *Francisella t larensis* which was cultured on blood agar supplemented with glucose (1 per cent) and cysteine (0.05 per cent) Y e. cultures were incubated at 25 C for 48 h, all other strains at 37 C for 24 h. The bacteria were harvested in saline, collected by centrifugation at 6,000 × *g* for 20 min, and kept at -20° C.

Preparation of Bacterial Extracts

All strains were suspended in phosphate buffered saline, pH 7.2 (PBS) 4 ml per gram bacteria (wet weight) and heated at 100° C for 60 min. The suspension was centrifuged at 6,000 × *g* for 20 min. The supernatant was dialysed against water for 2 days and lyophilized (heat extract)

CA was prepared from *S typhimurium* as reported by *Suzuki et al.* (14) In short, heat extract from the bacterium was mixed with absolute ethanol, 1:8.5 (v/v) The mixture was kept at 20 C for 18 h and the precipitate formed was removed by centrifugation. The supernatant was allowed to evaporate to dryness. The dried material was dissolved in distilled water dialysed against water and lyophilized (CA extract) Ethanol-soluble and -insoluble fractions from the Y e. extracts were obtained by the same technique. The fractions were dialysed and lyophilized

Immunization

Rabbits were immunized with suspensions of *E. coli* O 14, Y e. 3 or Y e. 9 bacteria adjusted to the an absorbance of 0.6 at 540 mμ with 10 mm cuvette Bacteria treated with formalin (0.3 per cent) or heated at 100° C for 60 min were used. I jections of 0.2, 0.4 0.6 and 1 ml were given intravenously on day 0, 3 7 and 10 respectively The ethanol-soluble fraction of the Y e. 9 heat extract was injected intravenously into the rabbit on day 0, 12, 20 and 30, in amounts ranging from 0.5 to 2 mg of the material in PBS. All animals were bled 7 days after the last injection. Sera were stored at -20 C and heated at 56 C for 30 min before testing.

Serological Methods

Sensitization of erythrocytes. Sheep erythrocytes were washed, packed and used for sensitization with the various preparations as described (11) The sensitizing activity of the antigens was determined by checkerboard titration using the appropriate antiserum. In the experiments, doses of 0.25 up to 1.5 mg of the various preparations was used for sensitization of 1 ml of 1.5 per cent suspension of erythrocytes. These amounts corresponded to 4 times the minimum amount needed to obtain maximum titre of antiserum.

Indirect haemagglutination test. Serum was absorbed with sheep erythrocytes and two-fold dilutions in 0.025 ml volumes of PBS were prepared using the microtitre equipment (Flow Laboratories, Irvine, Scotland) An equal volume of the suspension of sensitized erythrocytes was added. Plates were incubated at 4 C for 20 h. The agglutination was recorded by observing the pattern formed by the sedimented erythrocytes. Titre of serum is defined as the reciprocal of the highest serum dilution showing definite agglutination. This amount of serum corresponds to one agglutinating unit of antibody Sensitized erythrocytes in PBS and serum mixed with unsensitized cells served as controls.

Test for inhibition of indirect haemagglutination. Preparations to be tested for inhibition were diluted in 0.025 ml volumes of PBS and antiserum containing 4 agglutinating units of antibody was added. After incubation at 20° C for 30 min, 0.025 ml of the suspension of sensitized erythrocytes was added. Incubation and reading of the agglutination were as described. The minimum concentration (MIC) of the preparation (in mg per ml) giving complete inhibition of agglutination was determined.

Absorption of serum. Two mg of the bacterial extracts were mixed with 0.4 ml of serum diluted 1:16 The mixture was incubated at 4 C for 20 h and centrifuged at 1,000 × *g* for 20 min. The supernatant was tested for antibody activity

RESULTS

The titres in indirect haemagglutination test of sera from rabbits immunized with Y e. 3 Y e. 9 bacteria or *E. coli* O 14 are shown in Table 1 Antisera to the Y e. strains had titres of 512 or 1024 against erythrocytes sensitized with heat extract from the corresponding strain. These antisera also showed weak cross-reactivity with antigen from the Y e. strain of different serotype, but showed no activity against erythrocytes sensitized with the CA extract. However erythrocytes sensitized with CA and those sensitized with any

TABLE 1 *Titres of Rabbit Antisera to Y. enterocolitica (Y. e.) serotypes 3 and 9 and E. coli O 14 Tested against Erythrocytes Sensitized with Heat Extract from the Y. e. strains or with Common Enterobacterial Antigen (CA)*

Rabbit No.	Immunized with	Treatment of bacteria	Erythrocytes sensitized with		
			Y. e. 3 extract	Y. e. 9 extract	CA
399	Y. e. 3	Formalin	1024	16	< 8
885	Y. e. 3	100° C, 60 min	512	16	< 8
397	Y. e. 9	Formalin	16	512	< 8
884	Y. e. 9	100° C, 60 min	32	512	< 8
481	*E. coli* O 14	Formalin	1024	1024	1024
482	*E. coli* O 14	100° C, 60 min	512	512	512

TABLE 2. *Preparations Examined for Inhibition of Indirect Haemagglutination. Minimum Inhibiting Concentration is Given in mg/ml*

Source of preparation	Fraction tested for inhibition	Test system employed		
		CA extract + anti-E. coli O 14	Y. e. 3 extract + anti-Y. e. 3	Y. e. 9 extract + anti-Y. e. 9
Y. e. 3	Whole extract	0.25	0.125	> 4
	Ethanol-soluble	0.125	> 4	> 4
	Ethanol-insoluble	0.125	0.062	> 4
Y. e. 9	Whole extract	0.125	> 4	0.25
	Ethanol-soluble	0.25	> 4	> 4
	Ethanol-insoluble	0.062	> 4	0.125
S. typhimurium	CA extract	0.125	> 4	> 4

of the Y. e. heat extracts were agglutinated by antiserum to *E. coli* O 14. Serum drawn before immunization showed no antibody activity when tested in 1:8 dilution. The anti-CA antibodies could be removed by absorption of antiserum to *E. coli* O 14 with the CA extract or with any of the Y. e. heat extracts.

Whole extracts from the Y. e. strains, the ethanol-soluble and insoluble fractions of these extracts and the CA preparation were tested for inhibition of indirect haemagglutination (Table 2). All preparations examined showed inhibition of the activity of antibody to CA. Agglutination by the anti-Y. e. antisera of erythrocytes sensitized with the Y. e. heat extracts could be inhibited by whole extract and the ethanol-insoluble fraction from the same Y. e. strain. No inhibition was shown by the ethanol-soluble fraction, the CA extract, or any of the preparations originating from the other Y. e. strains.

Antisera agglutinating erythrocytes sensitized with any of the Y. e. heat extracts (see Table 1) also agglutinated those sensitized with the ethanol-insoluble fraction from the same extract. However erythrocytes sensitized with ethanol-soluble fractions were agglutinated only by antiserum to *E. coli* O 14 and not by any of the Y. e. antisera. Rabbit antiserum against the ethanol-soluble fraction of the Y. e. extract had a titre of 256 in tests with erythrocytes sensitized with the CA extract as well as in tests with those sensitized with any of the extracts or fractions from the Y. e. strains.

Heat extracts were prepared from five other Y. e. strains and from some other bacteria and tested for inhibition of antibodies to CA. The results listed in Table 3 show that extracts from strains of the various Y. e. serogroups and from Y. *pseudotuberculosis* had the ability to neutralize antibodies to CA.

TABLE 3. *Heat Extracts from Various Bacteria Tested for Inhibition of Agglutination of Erythrocytes Sensitized with CA by Anti-CA Antibodies*

Strain examined	Minimum inhibiting concentration (mg/ml)
Y. enterocolitica serogroup 1, 2a, 3 (Wauters)	0.5
13, 7	0.5
4, 33 "	0.5
5	0.5
6, 30	0.5
Y. pseudotuberculosis type I	0.25
E. coli O 14	0.125
Pasteurella multocida	> 4
Francisella tularensis	> 4
Brucella abortus	> 4
Acinetobacter calcoaceticus	> 4
Pseudomonas aeruginosa	> 4

Antibody-neutralizing activity was not shown by extracts from any of the other bacteria listed in Table 3. These bacteria also failed to remove antibodies against CA by absorption. Absorption of serum with extracts from any of the Y. e. strains examined or with that from Y. *pseudotuberculosis* depleted serum of anti-CA antibodies.

DISCUSSION

Rabbit antisera against *E. coli* O 14 contained antibodies which agglutinated erythrocytes sensitized with the CA extract and those sensitized with heat extract from the Y. e. 3 or Y. e. 9 bacteria. Absorption of the antisera with the Y. e. extracts removed the anti-CA antibodies, the activity of which was also inhibited by the extracts. These findings indicate that CA was present in the Y. e. heat extracts, in accordance with the observations by *Le Minor et al.* (8). Traditionally bacteria have been classified as belonging to the family *Enterobacteriaceae* on the basis of morphological, cultural and biochemical characteristics. However it has been suggested that demonstration of CA may also be used for taxonomic purposes (8, 18): bacteria producing CA belonging to the *Enterobacteriaceae*. The present demonstration that Y. e. strains of different serotypes or groups and Y. *pseudotuberculosis* produce CA puts further emphasis on the relationship between *Yersinia* and other genera of the *Enterobacteriaceae*.

Antibodies to CA could not be detected in serum from rabbits immunized with Y. e. 3 or Y. e. 9 bacteria. It therefore would appear that members of the species Y. *enterocolitica* do not induce CA antibodies in rabbits, analogous to the great majority of the *Enterobacteriaceae* (7, 17). This has been attributed to interference with production of these antibodies by the lipopolysaccharide (17) or its lipid A component (15). However immunogenicity of CA from Y. e. was demonstrated by immunization of rabbits with the ethanol-soluble fraction of the heat extract, similar to ethanol-soluble CA from other *Enterobacteriaceae* (17).

It was assumed that the agglutination of erythrocytes sensitized with the heat extracts by rabbit antisera was caused by antibodies against the corresponding *Yersinia* O antigen, analogous to agglutination by antibody against the O antigen from other Gram-negative bacilli (12, 13). This antigen could not be detected in the ethanol-soluble fraction of the heat extracts which contained CA. On the other hand, both CA and the O antigen were present in the ethanol-insoluble fraction, the antigens probably being closely associated (5, 10). Therefore, any of the preparations which sensitized erythrocytes for agglutination by antibody against the O antigen also sensitized

these cells for agglutination by anti-CA antibodies. It is apparently necessary in indirect haemagglutination tests designed to detect antibodies against the Y e. O antigen, to consider the possibility that agglutination is due to antibody to CA. This possibility was not considered in a recent report (9) Immunofluorescent antibody technique has been used both for serological investigation of Y e. strains (3 6) and for the detection of Y e. in clinical specimens (4) It has been shown that, in this test, antibodies to CA have the ability to stain bacteria that contain CA (1) Therefore it may be important to ensure that anti-CA antibody is not present in sera used for such testing

The role played by human serum antibodies against CA in tests designed to detect antibody to the Y e. O antigen is currently being investigated.

REFERENCES

1 *Aoki, S Merkel M & McCabe W R.*, Immunofluorescent demonstration of the common enterobacterial antigen. Proc. Soc. exp. Biol. (N Y) *121* 230–234 1966
2. *Bergey's Manual of Determinative Bacteriology* 8. ed. The Williams & Wilkins Company Baltimore, 1974
3 *Cederberg, A.:* The immunological specificity of fluorescent anti-*Yersinia enterocolitica* globulin. Acta path. microbiol. scand. *71* 376–386 1967
4 *Cederberg, A.*, Demonstration of *Yersinia enterocolitica* by the fluorescent antibody technique. Acta path. microbiol. scand. *73* 646–652, 1968.
5 *Hammarström S Carlsson H E. Perlmann, P & Svensson S* Immunochemistry of the common antigen of *Enterobacteriaceae* (Kunin) J exp. Med. *134* 565–576, 1971
6. *Karlsson K A., Hurvell, B. & Thal, E.* On the detection of *Yersinia enterocolitica* by the immunofluorescent method. In Winblad, S. (Ed.): Yersinia, Pasteurella and Francisella, vol 2 of Contributions to Microbiology and Immunology S. Karger Basel 1973, pp. 71–75.
7 *Kunin C M., Beard M F & Halmagyi, N. E.* Evidence for a common hapten associated with endotoxin fractions of *E. coli* and other *Enterobacteriaceae* Proc. Soc. exp. Biol. (N Y) *111* 160–166, 1962.
8. *Le Minor L. Chalon A-M & Véron M* Recherches sur la présence de l'antigène commun des Enterobacteriaceae (antigène Kunin) chez les *Yersinia Levinea, Aeromonas* et *Vibrio* Ann. Inst. Pasteur *123* 761–774, 1972.
9 *Lysy J & Knapp W.:* Serological studies of *Y enterocolitica*. In Winblad, S. (Ed.): Yersinia, Pasteurella and Francisella, vol. 2 of Contributions to Microbiology and Immunology S. Karger Basel 1973, pp. 42 53.
10. *Mayer H Schmidt G., Whang H Y & Neter E.:* Biochemical basis of the immunogenicity of the common enterobacterial antigen. Infect. Immunity *6* 540–544 *1972*.
11 *Meland J A.*, Antibodies in human sera against antigens in gonococci, demonstrated by a passive haemolysis test. Acta path. microbiol. scand. *67* 102–110, 1966.
12 *Neter E.*, Bacterial hemagglutination and hemolysis. Bact. Rev *20* 166–188, 1956.
13 *Neter E. Bertram L. F., Zak, D A., Murdock M R. & Arbesman C E.:* Studies on hemagglutination and hemolysis by *Escherichia coli* antisera. J exp. Med. *96:* 1 15, 1952.
14 *Suzuki T., Gorzynski E. A. & Neter E* Separation by ethanol of common and somatic antigens of *Enterobacteriaceae* J Bact. *88*. 1240–1243 1964
15 *Whang, H Y., Lüderitz, O., Westphal, O. & Neter E.* Inhibition by lipoid A of formation of antibodies against common antigen of *Enterobacteriaceae* Proc. Soc. exp. Biol. (N Y) *120* 371–374, 1965
16. *Whang H Y Mayer H Schmidt, G. & Neter E.* Immunogenicity of the common enterobacterial antigen produced by smooth and rough strains. *Infect.* Immunity *6* 533–539 1972.
17 *Whang, H Y & Neter E.*, Further studies on effect of endotoxin on antibody response of rabbit to common antigen of *Enterobacteriaceae* J Immunol. *98* 948–957 1967
18. *Whang, H Y & Neter E.*, Production of common enterobacterial antigen by members of the family *Enterobacteriaceae* Experientia (Basel) 29 96–97 1973.

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THE USE OF PROTEIN A-CONTAINING STAPHYLOCOCCI SENSITIZED WITH ANTI MENINGOCOCCAL ANTIBODIES FOR GROUPING *NEISSERIA MENINGITIDIS* AND DEMONSTRATION OF MENINGOCOCCAL ANTIGEN IN CEREBROSPINAL FLUID

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Olcén, P. Danielsson, D. & Kjellander, J. The use of protein A-containing staphylococci sensitized with anti-meningococcal antibodies for grouping *Neisseria meningitidis* and demonstration of meningococcal antigen in cerebrospinal fluid. Acta path. microbiol. scand. Sect. B, 83 387–396 1975.

The co-agglutination technique, utilizing antibody coated protein A-containing staphylococci, was successfully adapted to grouping *N. meningitidis* strains. It was found to give more clear-cut results than the standard slide test, especially in the case of strains isolated from throat specimens. The co-agglutination technique has also other advantages over the standard slide test in the grouping of meningococci: minor influence by auto-agglutination, higher specificity, easy performance and low consumption of specific antisera. Preliminary results also showed that the co-agglutination technique could be applied for the rapid detection of meningococcal antigen in cerebrospinal fluid.

Key words: *Neisseria meningitidis* grouping; meningococcal antigen; protein A-containing staphylococci.

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Dopter was in 1909 the first to show antigenic differences between meningococcal (MC) strains (11) and, a few years later *Gordon & Murray* described four serogroups (14). Since then, serological grouping of MC has been used for epidemiological purposes and was also used for therapeutic guidance in the pre-antibiotic era. Several classifications have been used which were reviewed by *Branham* in 1953 (3). The Committee on the Family Neisseriaceae of the International Committee on Bacteriological Nomenclature published in 1954 the recommended classification of MC into four serological groups, A, B, C and D (28) which is now generally accepted. *Slaterus* published in 1961 the finding of another three MC groups which he named X, Y and Z (27). This finding was verified by *Hollis et al.* (16)

25*

Evans et al. have described a further two groups named 29E and 135 (12)

Various serological techniques have been used for grouping slide agglutination, tube agglutination, capsular swelling, complement fixation test, "halo" formation in agar gel and double diffusion in agar (4 2 21 22, 15) The slide agglutination test is the standard technique most commonly used. It is a general experience, however, that many MC strains are not groupable by this technique because of spontaneous agglutination in specific antisera as well as in control sera.

Recently a new agglutination technique utilizing protein A-containing staphylococci sensitized with specific antibodies was described by *Kronvall* who used it for the typing of pneumococci (19) This technique, co-agglutination (COA) has also been adopted in the case of serological grouping of streptococci (5) mycobacteria (18) and is used for the identification of *N gonorrhoeae* (10)

A main advantage of the COA technique is that spontaneous agglutination is easily distinguished from true co-agglutination. In the present paper the COA technique has been adopted for the grouping of MC. Preliminary experiments are also presented where the technique has been used for the demonstration of MC antigen in cerebrospinal fluid (CSF) from patients with MC meningitis.

MATERIALS AND METHODS

Meningococcal Strains

International reference strains of MC representing serogroups A (NCTC No. 10025 batch 7) B (NCTC No. 10026 batch 3) C (NCTC No. 8554 batch 3) D (NCTC No. 9714 batch 2) X (NCTC No. 10790, batch 1) Y (NCTC No. 10791 batch 1) and Z (NCTC No. 10792 batch 1) were obtained from the National Collection of Type Cultures, Colindale, London. These reference strains were used for the production of polyvalent and group specific anti MC sera and for checking the specificity of the standard slide agglutination (SSA) and the co-agglutination (COA) systems. Fourteen MC strains, freshly isolated in our laboratory were used for serogrouping with the SSA and COA systems. Seven of these strains were isolated from CSF of patients with MC meningitis, the others from throat specimens routinely sent to the laboratory Five MC strains, ungroupable with SSA, were obtained from Dr. *Fallon*, Glasgow, Scotland. Another twelve MC strains freshly isolated from throat specimens were used for grouping with the COA system. Diagnostic criteria for MC were as follows: Gram-negative diplococci forming typical oxidase positive colonies that fermented glucose and maltose, but not levulose and lactose. Incapability to ferment lactose was ascertained through the ONPG test (6) The strains were preserved at –63 C in Trypticase Soy Broth with yeast extract and granulated agar

Other Bacterial Strains

Fifty-one bacterial strains belonging to species known to cause meningitis were tested for reactions with anti MC serum in the COA system. They represented the following bacterial species 12 strains of *Haemophilus influenzae* (three isolated from CSF) 14 strains of *Streptococcus pneumoniae* (two isolated from CSF and two from blood) seven strains of *Escherichia coli* (one isolated from CSF) eight strains of *Listeria monocytogenes* (one isolated from CSF) four strains of haemolytic group A streptococci and six strains of group B streptococci.

Culture of Strains

MC strains to be tested by the SSA technique were cultured according to Difco s directions. The same medium was used for COA tests. These were also performed after culture of the MC strains on GC agar medium made up from GC Agar Base supplemented with BBL haemoglobin and BBL Iso-vitalex. SSA as well as COA tests were performed after culture of the strains for 18–24 h at 35–37 C in an atmosphere containing 5 per cent CO_2. COA tests were also carried out after culture for 8–10 h.

The other bacterial strains, subjected to COA tests, were cultured on ordinary blood agar medium or on GC agar medium.

Cerebrospinal Fluid (CSF)

One hundred and seventeen CSF specimens routinely sent to our laboratory for bacteriological culture were examined. Eighteen specimens were from patients with bacterial meningitis, seven due to *N meningitidis* seven to *H influenzae* three to *Streptococcus pneumoniae* and one to *E coli*. The other 99 specimens were from patients with viral meningitis or from patients with other diseases. The specimens were examined for bacteria by Gram-stain, cultured according to standard procedures and subjected to co-agglutination tests as described below In some instances they were also



Fig. 1 Co-agglutination of *N meningitidis* group B using uncoated protein A-containing staphylococci (Cl) Cl coated with anti MC group A antibodies (Cl-anti MC A) and Cl coated with anti MC group B antibodies (Cl-anti MC B) MC B has been specifically co-agglutinated by Cl-anti MC B.

monitored by immunofluorescence techniques using reagents for *N meningitidis*, *H influenzae* and *Streptococcus pneumoniae*

Soluble Protein A

Lyophilized protein A, prepared as described by *Sjöquist et al.* (26) was kindly obtained from Dr *A Forsgren* and dissolved in distilled water at a concentration of 1 mg/ml.

Standard Slide Agglutination (SSA)

1) *Anti MC sera* were purchased from Difco laboratories, USA, and from the Pasteur Institute, Paris, France. One of the commercial reagents covered individual serogroups A, B, C, D, X, Y and Z, and in addition two pools. Pool 1 (P1) covered serogroups A, B, C and D and pool 2 (P2) serogroups X, Y and Z. The other commercial reagent covered individual serogroups A, B and C. The two commercial sources will be refered to as I and II respectively

2) *Performance of SSA* was in accordance with the directions by the manufacturers. The slides were tilted for approximately two minutes and positive agglutinations were graded + + + (strong) + + (moderate) and + (weak)

Co-agglutination (COA)

1) *Preparation of anti MC sera.* These were obtained from rabbits immunized with formalin-treated whole MC cells of the international reference strains. Antisera against the individual serogroups A, B, C, D, X, Y and Z were produced as well as two pools of antisera, pool 1 (P1) covering serogroups A, B, C and D pool 2 (P2) covering serogroups X, Y and Z. For preparation of antigens, the strains were cultured on GC agar medium for 18–24 h, harvested in sterile 0 15 M saline, and treated with formalin as described by *Danielsson* (7) Immunization with all antigens except P1 and MC B was performed by four intravenous injections three to four days apart, using doses increasing from 0.25 to 2.0 ml. The immunization schedule was repeated once if the antibody activity was low Anti MC sera pool 1 and serogroup B were prepared by intramuscular injections of antigens with complete Freund' adjuvant (7) Five weeks later a series of i.v injections was given as described above. The rabbits were bled one week after the last injection. The sera were stored frozen at −20° C. After thawing, the sera to be used were preserved by adding 1 per cent (vol/vol) merthiolate 1 100 and kept at +4 C. Co-agglutination with reagents prepared from unabsorbed antisera regularly gave cross reactions with heterologous MC groups. These antisera were therefore absorbed by mixing 1 volume of serum with 1 volume of appropriate formalin-treated MC cells as described by *Danielsson & Kronvall* (10)

2) *Preparation of reagent staphylococci.* Stabilized protein A staphylococci (strain Cowan I of *S aureus* NCTC 8530) were cultured and prepared as described elsewhere (1 5, 19) Some of these preparations were kindly supplied by Dr *P Christensen*. For coating of the protein A-containing staphylococci, 1 ml of a 10 per cent suspension was added to 0.1 ml of rabbit anti MC serum. After mixing, the staphylococci were washed twice and suspended to 1 per cent in PBS containing 0.1 per cent sodium azide.

3) *Performance of COA* a) The bacteria, cultured as described above, were emulsified in PBS to a concentration of approximately 1 per cent. One drop of this suspension was mixed on a glass slide with one drop of the 1 per cent suspension of reagent staphylococci coated with anti MC antibodies. The slide was tilted about once a second for approximately 2 minutes while observed

TABLE 1 *Results of Grouping International Meningococcal Reference Strains (A to Z) with Pel tination Techniques. Two Different Ant*

MC strains	Uncoated	Cowan I reagent staphylococci (COA)								
		Coated with MC antibodies								
		P1	A	B	C	D	P2	X	Y	
A	—	+ + +	+ + +	—	—	—	—	—	—	
B	—	+ +	—	+ + +	—	—	—	—	—	
C	—	+ + +	—	—	+ + +	—	—	—	—	
D	—	+ + +	—	—	—	+ + +	—	—	—	
X	—	—	—	—	—	—	+ + +	+ + +	—	
Y	—	—	—	—	—	—	+ + +	—	+ + +	
Z	—	—	—	—	—	—	+ + +	—	—	+ +

NRS = normal rabbit serum.

TABLE 2 *Results of Grouping Meningococci Freshly Isolated from Cerebrospinal Fluid (M 1 to M Slide Agglut*

MC strains	Uncoated	Cowan I reagent staphylococci (COA)								
		Coated with MC antibodies								
		P1	A	B	C	D	P2	X	Y	Z
M 1	—	+ + +	—	+ + +	—	—	—	—	—	—
M 2	—	+ + +	+ + +	—	—	—	—	—	—	
M 3	—	+ + +	—	—	+ + +	—	—	—	—	—
M 4	—	+ + +	+ + +	—	—	—	—	—	—	—
M 5	—	+ + +	+ + +	—	—	—	—	—	—	—
M 6	—	+ +	+ +	—	—	—	—	—	—	—
M 7	—	+ + +	—	—	+ + +	—	—	—	—	—

NRS = normal rabbit serum.

with the naked eye. Controls were performed with reagent consisting of uncoated staphylococci. In a positive reaction, granular agglutinates were formed while the suspension cleared. Strong co-agglutinations were graded + + + and appeared within 20–30 tilts. Moderate reactions were graded + + and appeared at a somewhat lower rate. Weak reactions were graded +. They developed slowly and were not obvious till after 50–60 tilts. The test was read as negative if co-agglutination failed to develop within 2 minutes. Positive and negative reactions are illustrated in Fig. 1

b) CSF specimens were subjected to COA tests in two ways as follows

1) One drop of anti MC reagent staphylococci was mixed with one drop of CSF on a glass slide, tilted to and fro and observed for co-agglutination during two minutes. This test will be referred to as "preliminary COA for CSF"

2) Since it was found that some CSF specimens gave non specific reactions by this test, various procedures were tried to overcome this. Pre-incubation of CSF with soluble protein A was found effective. This was carried out in the following way. One drop of CSF was mixed with one drop of protein A solution (1 mg/ml) or distilled water for one minute after which two drops of reagent staphylococci were added. The slide was then tilted to and fro and observed for co-agglutination during two minutes. This test will be referred to as "confirmatory COA for CSF". Controls with un-coated staphylococci were performed in parallel tests.

RESULTS

A. Grouping of Meningococci with Co-Agglutinating Reagent Staphylococci

Protein A-containing staphylococci coated with absorbed anti MC antibodies gave spe-

l Group-specific Anti Meningococcal Antibodies Using Co-Agglutination and Standard Slide Agglu
ed for Groups A B and C in the Standard SlSd Test

	Standard slide agglutination (SSA)								
NRS	MC antisera								
	P1	A	B	C	D	P2	X	Y	Z
—	+++	+++/+++	—/—	++/—	—	—	++	—	—
—	—	—/—	—/+++	—/—	—	—	—	—	—
—	+++	—/—	—/—	+++/+++	—	—	—	—	—
—	+++	—/—	—/—	—/—	+++	+	+	—	++
—	—	—/—	—/—	—/—	—	+++	+++	—	—
—	—	—/—	—/—	—/—	—	+++	—	+++	—
—	—	—/—	—/—	—/—	—	+++	—	—	+++

ith Polyvalent and Group-Specific Anti-Meningococcal Antibodies Using Co-Agglutination and Standard
Techniques

	Standard slide agglutination (SSA)								
NRS	MC antisera								
	P1	A	B	C	D	P2	X	Y	Z
++	++	++/—	++/+++	++/—	++	++	++	++	++
—	+++	+++/+++	—/—	+++/—	++	—	—	—	—
—	+++	—/—	—/++	+++/+++	—	—	—	—	—
—	+++	+++/+++	—/—	—/—	—	—	—	—	—
—	+++	+++/+++	—/—	—/—	—	—	—	—	—
—	+++	+++/+++	—/—	—/—	—	—	—	—	—
—	+++	—/—	—/—	+++/+++	—	—	—	—	—

cific and clear-cut COA reactions with the MC reference strains (Tabl 1) The same results were obtained whether the strains were cultured for 8–10 or 18–24 h on the medium used for standard agglutination or on GC agar medium.

Twenty two out of 24 MC isolated from clinical throat specimens were also specifically grouped with the COA test. One MC strain was extremely sticky and gave filamentous aggregates in all the reagents. The other MC strain gave ++ co-agglutination with CI-anti MC P2, but was negative with CI-anti MC X, Y and Z. The serogrouping of 7 of these MC isolated from throat specimens are presented below in the comparison of the SSA and COA tests.

B Comparison of Standard Slide Agglutination and Co-Agglutination for Grouping Meningococci

Reference MC strains and MC strains isolated from CSF and throat specimens were grouped by the standard slide agglutination technique using two commercial reagents for the groups A, B and C. Only one commercial reagent was available for groups D X, Y and Z and for pools 1 and 2. The SSA tests were performed according to the manufacturers' directions. The results were compared with those obtained by COA using absorbed anti MC antibodies (Tables 1–3)

TABLE 3 *Results of Grouping Meningococci, Freshly Isolated from Throat Specimens (TH 1 to TH* [cut off]
Slide Agglut

MC strains	Uncoated	Cowan I reagent staphylococci (COA) Coated with MC antibodies								
		P1	A	B	C	D	P2	X	Y	Z
TH 1	—	—	—	—	—	—	+ +	—	+ +	—
TH 2	—	—	—	—	—	—	+ +	—	+ +	—
TH 3	—	+	—	+ + +	—	—	—	—	—	—
TH 4	—	+ + +	—	—	+ + +	—	—	—	—	—
TH 5	—	—	—	—	—	—	+ +	—	+ +	—
TH 6	—	—	—	—	—	—	+ + +	—	+ + +	—
TH 7	—	—	—	—	—	—	+ +	—	+ +	—

NRS = normal rabbit serum.

1) Tests with MC Reference Strains

It will be seen from Table 1 that the COA tests were clear-cut. The same was true of some of the commercial reagents in the SSA test. However the polyvalent reagent P1 and one of the anti MC B sera failed to agglutinate MC B. One of the anti MC C sera gave also a moderate cross reaction with MC A. Similar cross agglutination reactions, weak or moderate, were also obtained with one or two heterologous MC groups, using the polyvalent anti MC serum P2, the anti MC X, and anti MC Z sera.

2) Tests with MC Strains Isolated from CSF

It will be seen from Table 2 that the seven MC strains, freshly isolated from CSF were all distinctly grouped by COA tests. As regards six strains, agreeing results were obtained by SSA tests using one of the commercial reagents and, as regards five strains, the other reagents would give such results. One strain (M 1) group B by the COA test and SSA test using the commercial reagent II was non-groupable with the other commercial SSA sera because of auto agglutination One strain (M 2) group A by the COA test and one SSA test also reacted with anti MC C and to some degree with anti MC D in the SSA test using reagent I. One strain (M 3) MC C by COA test and SSA test using reagent I reacted with anti MC C as well as anti MC B in the SSA test using reagent II

3) Tests with MC Strains Isolated from the Throat

Table 3 shows that the MC strains isolated from 7 throat specimens were groupable by COA tests. Agreeing results were obtained by SSA tests in the case of 2 of the strains, one using reagent I (TH 4) and the other reagent II (TH 3) As regards the other strains, the results obtained by the SSA tests were not clear-cut because of cross reactions between two or more groups. It is of interest to note that 3 strains (TH 1 TH 5, TH 7) grouped as MC Y by the COA test, did not react with anti MC Y sera in the SSA tests. The reason why is obscure since both COA and SSA tests gave clear-cut results if the international reference strains were used. Of practical value is the observation that, if only practical antisera against the three most common serotypes causing meningitis (A, B, C) had been used for grouping with the SSA test, 5 of the strains would have been (incorrectly) classified as group B or C whether one or the other reagent were used.

4) Tests with Auto Agglutinating MC Strains

Five strains obtained from Dr Fallon, Glasgow Scotland, were auto-agglutinating in SSA tests. In COA tests, however they were all typable and gave + + or + + + reactions with CI-anti MC P2 and CI-anti MC Y and negative reactions with the other reagents.

[…] Polyvalent and Group-Specific Anti-Meningococcal Antibodies Using Co-Agglutination and Standard […]chniques

RS	Standard slide agglutination (SSA)								
	MO antisera								
	P1	A	B	C	D	P2	X	Y	Z
—	+ +	—/—	—/—	+ +/—	+ +	—	+ +	—	—
·+	+ + +	+ +/+ + +	+ +/—	+ + +/+ +	+ +	+ +	—	+ +	—
—	—	—/—	—/+ + +	—/—	—	—	—	—	—
—	+ + +	—/—	—/+ +	+ + +/+ + +	—	—	—	—	—
—	+	—/ –	—/+ +	+ +/—	—	+ + +	—	—	+ +
—	+ +	—/+ +	+ + +/+ +	+ +/—	+	+ + +	+ +	+ +	+ + +
—	—	—/—	+ +/—	—/+ + +	—	+ + +	—	—	+ + +

C Use of Co-Agglutination for Rapid Diagnosis of Meningococcal Meningitis

Preliminary experiments showed that protein A-containing staphylococci coated with anti MC antibodies could be used to detect MC organisms or MC antigens in CSF These experiments were therefore extended as follows

1) Reagent staphylococci coated with anti MC P1 and P2 antibodies, appropriately absorbed with heterologus MC *strains*, were tested for specificity reactions against the bacterial species commonly isolated from CSF in patients with purulent meningitis (see Material and Methods) No co-agglutination reactions were obtained *except in the case of 4 E. coli strains with the capsular antigen K1* These strains gave a positive co-agglutination with Cl-anti MC P1 and Cl anti MC B, but not with any other reagent.

2) One hundred and seventeen CSF specimens, *sent to the laboratory for bacteriologi*cal culture, were subjected to preliminary COA tests as described under Materials and Methods.

Four CSF specimens gave clear-cut COA *reactions* (Fig 2) and MC were isolated from these specimens. Negative COA was noted in 85 CSF specimens, two of these with positive cultures of *Streptococcus pneumoniae* one with *H influenzae* and 82 with *negative cultures*. False positive COA reac

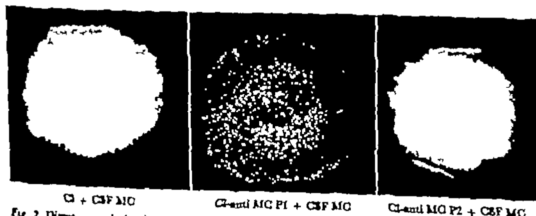


Fig 2. Direct co-agglutination of cerebrospinal fluid from a patient with meningitis due to MC group C (CSF MC) using uncoated protein A-containing staphylococci (Cl) Cl coated with pooled anti MC group A, B, C and D antibodies (Cl-anti MC P 1) and Cl coated with pooled anti MC group X, Y and Z antibodies (Cl-anti MC P 2) The CSF has been specifically co-agglutinated by Cl-anti MC P 1

TABLE 4 *Results of Co-Agglutination Tests of CSF before and after Treatment with Soluble Protein A*

CSF No.	Bacteria isolated	Untreated CSF			CSF pretreated with Protein A		
		Cl	Cl-anti MC P1	Cl-anti MC P2	Cl	Cl-anti MC P1	Cl-anti MC P2
1	None	—	+ +	+ +	—	—	—
2	None	—	+ +	—	—	—	—
3	None	—	+	—	—	—	—
4	None	—	+	—	—	—	—
5	*H infl.*	—	+ + +	—	—	—	—
6	*H infl.*	—	+	—	—	—	—
7	None	—	+	—	—	—	—
8	Pnc	—	+ +	+ +	—	—	—
9	*H infl.*	—	+ +	—	—	—	—
10	*H infl.*	+ + +	+ + +	+ + +	+ +	+ +	+ +
11	MC A	+	+ + +	+ +	—	+ + +	+ +
12	MC A	+	+ + +	+ +	—	+ + +	+ +
13	MC C	—	+ + +	+	—	+ + +	—

Cl = uncoated reagent staphylococci.
Cl-anti MC P1 = reagent staphylococci coated with anti MC A, B, C and D
Cl-anti MC P2 = reagent staphylococci coated with anti MC X, Y and Z.

tions with 28 CSF specimens, however were noted. Twenty-two of these showed no growth of bacteria. In four *H influenzae* was isolated, in one *Streptococcus pneumoniae* and in one *E. coli*. The *E. coli* strain possessed the capsular antigen K 1 The CSF in this case reacted with Cl-anti MC P1 only

It was found that "preliminary COA tests for CSF" giving false positive reactions could be ruled out by a "confirmative COA test" (see *Materials and Methods*) The results of such experiments on 10 available CSF's out of the 28 false positive ones are summarized in Table 4 As can be seen, the false positive reactions were eliminated while the specific ones persisted. In one CSF (no. 10) with a high leucocyte count and heavy growth of *H influenzae* the reactions persisted but occurred also if the control reagent was used. The running of a parallel test using CSF diluted 1.2 with distilled water ruled out that a positive preliminary but negative confirmative COA test could be due to CSF diluted by the addition of the protein A solution.

Three CSF specimens with growth of MC are also included in Table 4 to demonstrate that the pretreatment with protein A does not eliminate the specific COA reactions. MC group A was isolated from two of these specimens. Both gave a positive COA test with Cl-anti MC P1 as well as with Cl-anti MC P2. MC group C was isolated from the third specimen and this gave a positive COA test with Cl-anti MC P1 only

DISCUSSION

During recent years, increasing attention has been paid to serogrouping of *MC* isolated from patients with meningococcal disease as well as from carriers. For this purpose, the slide agglutination has been the standard technique. The majority of the clinical strains belong to groups A, B or C and they are usually groupable by this technique. Some strains, however are *auto-agglutinable* and other strains cannot be distinctly grouped because of cross reactivity These problems are especially pronounced in the case of carrier strains belonging to groups *other* than A, B or C. This has been documented in several reports and was also found in the present investigation.

In the present work it was demonstrated that the co-agglutination technique as de-

scribed by *Kronvall* (19) can be applied to the grouping of MC. The technique has several advantages over standard slide agglutination. It gives more clear-cut results and is not influenced by auto-agglutination. The same advantages have been demonstrated in other bacterial systems (5, 10). In addition, it is easy to perform and economic in use since 1/10 ml of specific antiserum is sufficient for the preparation of 10 ml of reagent staphylococci.

Although agreeing results were obtained by the COA and SSA techniques in tests of international reference strains and clinical isolates there were some discrepancies in the case of carrier strains (Table 3). An explanation of these discrepancies could be that the COA technique offers greater sensitivity and specificity since only IgG antibodies are adsorbed to protein A-containing staphylococci while IgG, IgM and possibly IgA antibodies may participate in SSA tests. It is also well known that MC have many antigens in common (3) and the COA tests seem to give a better discrimination between common and group specific antigens than antibodies operating in SSA tests. It is possible that the use of COA tests could lead to a re-evaluation of the serogrouping of MC, especially of carrier strains. This would be of value in epidemiological studies. Work is now in progress along these lines.

Various serological techniques such as precipitation (23), complement fixation (20), immunofluorescence (8, 9), counter immunoelectrophoresis (15) and latex agglutination (25) have been used for a rapid demonstration of MC or MC antigens in CSF from patients with meningitis. The results of the present work have shown that COA of CSF with specific reagent staphylococci could be an excellent adjunct to direct microscopy and culture in the diagnosis of meningococcal meningitis. Such a COA test takes less than 5 minutes to perform and, by including reagents also for *H. influenzae* and pneumococci, the most common bacterial pathogens in purulent meningitis would be covered. The possibilities of false positive reactions must, however, be considered since protein A staphylococci will be agglutinated in the presence of normal human serum (17). In CSF with elevated concentration of protein, an agglutination is also to be expected. This seems to have operated in some of the CSF specimens. However, pretreatment of CSF with soluble protein A eliminated the false positive reactions, probably by blocking IgG molecules (15) that would otherwise have reacted with the protein A staphylococci. The specific COA reactions persisted after CSF containing MC and/or MC antigens, had been pretreated with protein A.

It was found that some of the CSF's containing MC gave positive COA tests with the two pools of anti MC reagent staphylococci despite the fact that the MC strains after isolation by culture reacted specifically with one pool only. A probable explanation of this could be that MC in CSF were damaged, thus uncovering common MC antigens. Work is now in progress to elucidate this.

The noted reaction between *E. coli* containing K 1 antigen and anti MC B reagent staphylococci is of interest. The findings are in agreement with those recently reported by *Robbins et al.* (24) according to whom an antigenic relationship between MC B and the K 1 antigen exists. This *E. coli* strain has been isolated in newborn infants with *E. coli* meningitis. A COA test of a CSF specimen could thus give a false positive reaction, but this can be avoided by making a Gram stain and a morphologic examination at the same time.

In the present paper, MC strains confirmed by bacteriological and biochemical tests were subjected to serological grouping using the COA technique. Preliminary studies also indicate that this technique could be used instead of time consuming biochemical tests to discriminate MC from other neisseria. Work is now in progress along these lines.

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REFERENCES

1. *Arvidson, S., Holme T & Wadström T* Formation of bacteriolytic enzymes in batch and continuous culture of *Staphylococcus aureus*. J Bacteriol. *104*: 227–235 1970
2. *Beckler E. A.*: Meningococcus grouping. Note on experience with the capsular swelling test. J Lab. Clin. Med. *30* 745–747 1945
3. *Branham S E.*: Serological relationships among meningococci. Bacteriol. Rev *17* 175–188, 1953
4. *Branham S E.* Reference strains for the serologic groups of meningococcus (*Neisseria meningitidis*) Intern. Bull. Bact. Nom. and Tax. *8* 1–15 1958.
5. *Christensen, P., Kahlmeter G., Jonsson S & Kronvall, G.*. New method for the serological grouping of streptococci with specific antibodies adsorbed to protein A-containing staphylococci. Infect. Immun. 7 881–885 1973
6. *Corbett W P & Catlin B. W.*. Galactosidase activity of lactose-positive Neisseria. J Bacteriol. *95* 52–57 1968.
7. *Danielsson D* The demonstration of *N gonorrhoeae* with the aid of fluorescent antibodies. 1 Immunological studies of antigonococcal sera and their fluorescein-labelled globulins, with particular regard to specificity Acta Derm. Venerol. *43* 451–464 1963
8. *Danielsson D & Wåhlin S* Diagnostik av meningokockmeningiter med immunofluorescens. Svenska Läkartidningen *64* 4364–4368 1967
9. *Danielsson D Nathorst Windahl, G & Saldén T.*. Use of immunofluorescence for identification of *Haemophilus influenzae* and *Neisseria meningitidis* in postmortem human tissue. Ann. NY Acad. Sci *177* 23–31 1971
10. *Danielsson D & Kronvall, G* Slide agglutination method for the serological identification of *Neisseria gonorrhoeae* with anti-gonococcal antibodies adsorbed to protein A-containing staphylococci. Appl. Microbiol. *27* 368–374 1974
11. *Dopter Ch.* Etude de quelques germes isolés du rhino-pharynx, voisins du méningocoque (paraméningocoques) Compt. rend. soc. biol *67* 74–76 1909
12. *Evans J R., Artenstein, M S & Hunter D H* Prevalence of meningococcal serogroups and description of three new groups. Am. J Epidemiol. *87* 643–646 1968.
13. *Forsgren A. & Sjöquist J.*. "Protein A" from *S aureus*. I Pseudo-immune reaction with human γ-globulin. J Immunol. *97* 822–827 1966.
14. *Gordon M H & Murray E. G. D* Identification of the meningococcus. J Roy Army Med. Corps. *25* 411–423, 1915.
15. *Greenwood B. M., Whittle H C & Dominic Rajkovic O.*: Counter-current immunoelectrophoresis in the diagnosis of meningococcal infections. Lancet: 519–521 Sept. 4 1971
16. *Hollis D G., Wiggins, G. L. & Schubert, J H.*: Serological studies of ungroupable *Neisseria meningitidis*. J Bacteriol. *95*: 1-4 1968
17. *Jensen, K.* Undersøgelser over stafylokokkernes antigenstruktur Thesis. Ejnar Munksgaard. Copenhagen 1959.
18. *Juhlin I & Winblad, S* Sero-typing of mycobacteria by a new technique using antibody globulin adsorbed to staphylococcal protein A. *Acta path. microbiol. scand. Sect. B*, *81* 179–180 1973
19. *Kronvall G.*. A rapid slide-agglutination method for typing pneumococci by means of specific antibody adsorbed to protein A-containing staphylococci. J Med. Microbiol. *6* 187–190, 1972.
20. *Macgrath, B. G* The diagnosis of meningococcal meningitis from the spinal fluid. Lancet 17 19 Jan 6, 1934
21. *Nicolle M Debains E. & Jouan, C.*: Etudes sur les méningocoques et les sérums antiméningococcoques. Ann. Inst. Pasteur *32*: 150, 1918.
22. *Petrie G F* A specific precipitin reaction associated with the growth on agar plates of meningococcus, pneumococcus and *B. dysenteriae* (Shiga) Br J Exp. Pathol. *13* 380–394, 1932.
23. *Rake G* Studies on meningococcus infection. V The presence of meningococcus precipitinogens in the cerebrospinal fluid. J. Exp Med *58* 375–383 1933.
24. *Robbins J B., McCracken G H Gotschlich, E. C., Orskov F Orskov I & Hanson L A.* Escherichia coli K1 capsular polysaccharide associated with neonatal meningitis. N Engl. J Med *290* 1216–1220, 1974
25. *Severin, W P J* Latex agglutination in the diagnosis of meningococcal meningitis. J Clin. Path. *25* 1079–1082, 1972.
26. *Sjöquist J Meloun B. & Hjelm H* Protein A isolated from *Staphylococcus aureus* after digestion with lysostaphin. Eur J Biochem *29* 572–578, 1972.
27. *Slaterus K. W.*. Serological typing of meningococci by means of micro-precipitation. Antonie van Leeuwenhoek *27* 305–315 1961
28. Preliminary report of the committee on the family Neisseriaceae. Internat. Bull. Bact. Nomen. and Tax. *4* 95–105, 1954

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PURINE METABOLISM IN *NEISSERIA MENINGITIDIS*

3. *Utilization of Exogenous Hypoxanthine, Guanine and Xanthine*

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Jyssum, S. Purine metabolism in *Neisseria meningitidis*. 3. Utilization of exogenous hypoxanthine, guanine and xanthine. Acta path. microbiol. scand. Sect. B, 83 397–406, 1975

Whole cells of *Neisseria meningitidis* have been found to utilize exogenous radioactive hypoxanthine, guanine and xanthine. When hypoxanthine was the precursor, the pools of both the adenine and the guanine 5'-ribonucleotides were labelled. Guanine and xanthine were utilized with labelling of the pool of the guanine 5'-ribonucleotides only. Crude extracts from *N. meningitidis* were found to have activities corresponding to hypoxanthine phosphoribosyltransferase (EC 2.4.2.8.) and another phosphoribosyltransferase which seems to exhibit specificity for guanine and xanthine. Crude extracts phosphorylated guanosine 5'-monophosphate to guanosine 5'-triphosphate in the presence of adenosine 5'-triphosphate (ATP) and $MgCl_2$.

Key words: *Neisseria meningitidis*; purine metabolism.

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In a previous paper it was shown that the purine mutant 6-1b of *N. meningitidis* requiring the amino acids histidine and proline and the purine bases adenine and guanine for growth could grow with hypoxanthine as the only purine source, whereas xanthine could substitute for guanine (12). *N. meningitidis* extracts were shown to have activities corresponding to adenine phosphoribosyltransferase, EC 2.4.2.7 (12). Phosphoribosyltransferases (PRTases) which catalyze the condensation of purine bases and 5-phospho-α-D-ribose 1-diphosphate (PRPP) to form their respective mononucleotides (17, 18, 20) are widely distributed in nature. Mammals have two such enzymes. One has specificity for 6-aminopurines (adenine phosphoribosyltransferase EC 2.4.2.7.) (6, 17) and the other for 6-oxopurines (hypoxanthine phosphoribosyltransferase EC 2.4.2.8) (6, 20). In bacterial cells, the patterns of activities are more diverse. *Escherichia coli* B (18, 24) and *Salmonella typhimurium* (5, 8) have an enzyme which converts both guanine and xanthine to their mononucleotides, while an enzyme from *Brevibacterium ammoniagenes* (26) has been reported to act on both adenine and xanthine. A phosphoribosyltransferase which is specific for xanthine has been observed in *Streptococcus faecalis* (3, 23), *S. typhimurium* (14), *Bacillus subtilis* (2) and *Lactobacillus casei* (18).

In the present work, the labelling of purine intermediates from radioactive hypoxanthine, guanine and xanthine taken up by whole

cells has been examined. Subsequently the enzyme activities in crude extracts toward hypoxanthine, guanine and xanthine have been measured. The activity toward hypoxanthine was distinct from the activities toward guanine and xanthine. This was shown by the differences in rates of heat inactivation, and in the stability of the enzymes toward dialysis and freezing.

MATERIALS AND METHODS

Strains. N meningitidis strain M1 of group B was, as before, the test microbe (12) *E. coli* K12 was used in one experiment for comparison (12, 13)

Media. Blood agar plates were used as solid media. Fluid medium was the enriched, defined Medium KC (13) Fluid minimal medium for *E. coli* was that of *Davis & Mingioli* (7)

Growth conditions N meningitidis (13) and *E. coli* (12) were grown as described previously

Cell free extracts. Overnight cultures on blood agar were used for preparing cell free extracts for the enzyme assays (11) The cells, 0 1 to 0.2 g wet weight, were suspended in 1 ml 0.05 M Tris/HCl buffer at pH 7 4 before ultrasonic disintegration (11) After centrifugation, the supernatants were diluted with 15 parts of 0.05 M Tris/HCl buffer pH 7 4 if not otherwise stated. These diluted extracts were used immediately for the enzyme assays unless otherwise stated. Protein was estimated by the procedure of *Lowry et al.* (21)

Assay for labelled intermediates in intact cells. The separation, identification and estimation of radioactive metabolites were as described previously (12)

Purine phosphoribosyltransferase The enzyme activity was assayed in a total volume of 50 μl by mixing 4 μmoles Tris/HCl or K-phosphate buffer pH 7 4 (37 C) (stated in the actual experiment) 0.05 μmole $MnCl_2$ or 0.5 μmole $MgCl_2$ (stated in the actual experiment) 0.05 μmole PRPP-tetrasodium salt 5 nanomoles of one of the following bases ^{14}C-8-hypoxanthine (62 μCi/μmole) ^{14}C-8-guanine (56 μCi/μmole) C-2 xanthine (48 μCi/μmole) C-8-adenine (54.2 μCi/μmole) and 10 μl extract. The reaction was initiated by the addition of PRPP and carried out at 37 C in a shaking water bath. PRPP was handled as previously described (12) In most of the experiments, the reaction was run for 10 minutes and terminated by cooling in an icewater bath. The protein was precipitated with 50 μl ice-cold 96 per cent ethanol, followed by 5 μl 0 1 M potassium-EDTA, and removed by centrifugation. Appropriate controls with deproteinized extracts were included. Separation of the purine bases from their respective monophosphates was achieved as previously described (12) by chromatography of 5 μl of the supernatants on cellulose thin layers (20 × 20 cm) (Eastman Chromagram with fluorescent indicator) for 6 to 7 cm in 1M NH_4 acetate pH 7.0. The thin layer origins were spotted with unlabelled purine bases and monophosphates to facilitate detection of spots under ultraviolet light (12) The spots were marked by a pencil, and strips from the position of the monophosphate to the solvent front were cut and counted as before in the Packard Tri-Carb Spectrometer (12). The bases and their monophosphates have the following R_F in NH_4-acetate hypoxanthine (Hx) 0.55 inosine 5'-phosphate (IMP) 0.85 guanine (G) 0.34 guanosine 5'-phosphate (GMP) 0.75, xanthine (X) 0.59 xanthosine 5'-phosphate (XMP) 0 79 adenine (A) 0.31 and adenosine 5'-phosphate (AMP) 0.67 As a routine, XMP was spotted instead of IMP in the experiments with Hx since some experiments, if performed with undiluted new extracts, showed radioactivity corresponding to XMP amounting to about 2 per cent of the radioactivity in the IMP spot. This was confirmed by chromatography for 17 cm in the isobutyric acid solvent of *Krebs & Hems* (16) as used before (12) Hx, IMP and XMP have the R_F values of 0.65 0.30 and 0.19 respectively in this solvent on cellulose thin layers. Any XMP formed plus the IMP formed were then analysed. Any radioactive di- and tri-phosphates formed have a higher R_F value in NH_4-acetate than the monophosphates Radioactive GDP and GTP were not detected by this procedure even if active GMP and GDP kinases were present in crude extracts. If necessary the chromatograms were, after development, exposed to X-ray film for –4 days before counting of the spots (11) Enzyme activity is given as nanomoles monophosphate formed during the time interval indicated

Reaction mixture for estimation of the actual concentration of PRPP in solution. A total of 50 μl consisted of 4 μmoles Tris/HCl buffer pH 7.4 0 05 μmole $MnCl_2$ 0.05 μmole ^{14}C-8-hypoxanthine, adjusted to specific activity 9.9 μCi/μmole with the nonradioactive compound; 10 nanomoles PRPP by weight, and 10 μl undiluted extract. The rest of the procedure was as that used for purine phosphoribosyltransferase Correction was made for endogenous PRPP in the extracts.

Guanylate kinase EC 2. 4.8 (6) A reaction volume of 105 μl consisted of 10 μmoles Tris/HCl buffer pH 7.6 1 μmole $MgCl_2$; 0.5 μmole ATP 7.6 nanomoles ^{14}C-8-GMP (61 μCi/μmole) and 25 μl crude (not diluted) extract. The reactions were run for 5 10, 15 30 and 60 minutes at 37 C. Protein was precipitated at 0° C with 100 μl 96 per cent ethanol. After centrifugation 5 μl

of the supernatants were spotted for chromatography on cellulose thin layer for 17 cm in the n-butanol-acetone-acetic acid-conc. NH_3, H_2O (45:15:10:2:28, by vol.) solvent used previously (12). The chromatogram was developed twice in this solvent. This gave good separation of the ribonucleotides. The chromatogram was exposed to X-ray film before cutting and counting. The R_F of the metabolites are GMP 0.19, GDP 0.11 and GTP 0.07.

Chemicals ^{14}C-8-hypoxanthine (59 and 62 mCi/mmole), ^{14}C-8-guanine *sulphate-monohydrate* (36.1 and 56 mCi/mmole), ^{14}C-8-adenine (54.2 mCi/mmole), ^{14}C-8-guanosine 5'-monophosphate ammonium salt (61 mCi/mmole) were obtained from The Radiochemical Centre, Amersham, Bucks. U.K. ^{14}C-2-xanthine (48 mCi/mmole) was obtained from CEA, Department des Radioelements, 91-Gif-Sur-Yvette, France. The purity of the radioactive substrates was controlled as before (12). Hypoxanthine was found to contain 0.5 per cent adenine. This small amount was not found to disturb the results and the base was used as such. Other fine chemicals were obtained from Koch-Light Labs. Ltd. Bucks. U.K. or from Sigma Chemical Corp. St. Louis, Mo. U.S.A.

RESULTS

Labelling of purine metabolites from ^{14}C-8-hypoxanthine, ^{14}C-8-guanine and ^{14}C-2 xanthine. If whole cells of *N. meningitidis* were exposed to radioactive hypoxanthine, label appeared in the adenosine 5' nucleotides and the guanosine 5' nucleotides as shown in Table 1. If cells were exposed to radioactive guanine and xanthine, label appeared only in the guanosine 5' nucleotides (Table 1).

E. coli was exposed to radioactive guanine by way of comparison. In this microbe, label from guanine appeared in both the adenosine and the guanosine 5' nucleotide pools (Table 1). The 2-dimensional chromatographic system used (12) gave good separation of the adenosine 5'-nucleotides (AMP, ADP, ATP) and adenylosuccinate (S-AMP) from the guanosine 5' nucleotides (GMP, GDP, GTP), IMP and XMP. The separation of IMP, GMP and XMP varied from chromatogram

TABLE 1 *Labelling of Purine Metabolites from ^{14}C-8-hypoxanthine, ^{14}C-8-guanine and ^{14}C-2 xanthine in Intact Cells of* N. meningitidis *and* E. coli

Organism	Time of incubation sec	Purine base added		Incorporation calculated as picomoles purine base		
				Purine base	AMP + ADP + ATP	GMP + GDP + GTP
N. meningitidis						
Strain M1	15	Hypoxanthine	2.3 µg	1.1	120.3	62.6
	60	Hypoxanthine	2.3 µg	16.2	148.2	73.4
	120	Hypoxanthine	2.3 µg	0.8	182.3	128.5
N. meningitidis						
Strain M1	15	Guanine	2.5 µg	281.5	–	119.2
	60	Guanine	2.5 µg	525.8	–	132.8
	120	Guanine	2.5 µg	570.7	–	146.8
E. coli K12	120	Guanine	2.5 µg	104.6	78.1	50.7
N. meningitidis						
Strain M1	15	Xanthine	3.6 µg	0.8	–	45.7
	60	Xanthine	3.6 µg	0.8	–	63.7
	120	Xanthine	3.6 µg	0.9	–	73.0

Batches of 1 ml culture in exponential growth in Medium KC (*N. meningitidis*) and in that of *Davis & Mingioli* (*E. coli*) with appr. 1.5 × 10^9 colonyforming units were exposed to the amount of bases shown for the time indicated. The purine bases had the following specific activities: hypoxanthine (59 µCi/µmole), guanine (36.1 µCi/µmole), xanthine (48 µCi/µmole). The rest of the procedure as described before (12).

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to chromatogram, but only one radioactive spot was always seen on the films in this area, corresponding best to GMP More radioactivity was usually recovered in the adenosine and guanosine di-and tri-phosphates than in the monophosphates. Radioactivity corresponding to S-AMP was not seen. The turnover of the purine bases into the adenosine and guanosine phosphates must therefore be very rapid in proliferating cells (Fig 1)

N meningitidis does not seem to contain IMP or XMP kinase. In one experimental system, ^{14}C-hypoxanthine or ^{14}C-xanthine, K-phosphate buffer $MnCl_2$, PRPP and 25 μl undiluted meningococcal extract were incubated for 30 minutes at 37° C. In this system, all the hypoxanthine and xanthine was transformed to IMP and XMP respectively Upon addition of ATP and $MgCl_2$ and incubation for another 30 minutes, chromatography of the supernatants on thin layer cellulose in the isobutyric acid solvent (12, 16) and for control in NH_4-acetate, both for 17 cm and radioautography spots corresponding only to IMP and XMP were seen on the film when hypoxanthine was the substrate. If xanthine was used as substrate only one spot corresponding to XMP was seen.

Purine Phosphoribosyltransferase

Effect of enzyme concentration and time The enzyme activities toward hypoxanthine, guanine and xanthine were tentatively tested with potassium-phosphate buffer pH 7 4 and Mn^{++} (1mM) as cation in the reaction mixture (Table 2) since the highest activity of adenine phosphoribosyltransferase in crude meningococcal extracts was found under these experimental conditions (12) Table 2 shows that as much as 88 per cent of the substrate (guanine) was converted in 10 minutes when undiluted extract was used. Experiment no. 2 using diluted extract shows the enzyme activities related to time. Table 2 also shows that the activities toward hypoxanthine, guanine and xanthine were much higher than the activity toward adenine. Because of the high activities of purine phosphoribosyltransferases in crude extracts with hypoxanthine, guanine and xanthine as substrates the extracts were diluted as stated

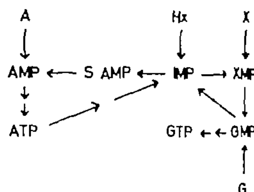


Fig 1 Conversion of purine bases to their monophosphates and interconversion of the ribonucleotides (22) A, adenine Hx, hypoxanthine; G, guanine X, xanthine AMP adenosine 5-phosphate ATP adenosine 5-triphosphate IMP inosine 5-phosphate S-AMP adenylosuccinate XMP xanthosine 5-phosphate GMP guanosine 5'-phosphate GTP guanosine 5-triphosphate. →→ more than one enzyme.

TABLE 2. *Purine Phosphoribosyltransferase Activities in Cell-free Extracts from* N. meningitidis

Expt. No.	State of extract	Protein µg/assay	Time of incubation min	^{14}C-purine base converted nanomoles Hx	G	X	A
1	New undiluted	70	10	3.37	4 41	5 14	0 22
2	New diluted	2.8	2	0.07	0 10	0.12	0 00
		2.8	6	0.25	0 39	0.36	0 01
		2.8	10	0.32	0 64	0.50	0 01

The enzyme activities were assayed in K-phosphate buffer pH 7 4 with Mn^{++} (1 mM) as cation. The rest of the procedure as described under Methods.

TABLE 3 *Purine Phosphoribosyltransferase Activities in Extract from* N meningitidis

Buffer used in assay pH 7 4	Cation and conc. of cation mM	[1] C-purine base converted nanomoles			Per cent activity		
		Hx	G	X	Hx	G	X
Tris	Mn^{++} 1	1 72	1.28	1.52	100	100	100
Tris	Mg^{++} 10	0 72	0 75	0.81	42	58	53
K-phosphate	Mn^{++} 1	0.89	0.56	0.91	5	43	60
K-phosphate	Mg^{++} 10	0.52	0 71	0.77	30	55	51

Experimental procedure as described under Methods. 10 µl extract contained 3.2 µg protein.

under Methods and the reactions were run for 10 minutes only to obtain experimental conditions that allowed comparison of the activities.

Effect of buffers and divalent cations The phosphoribosyltransferases showed the highest activities in Tris/HCl buffer and with Mn^{++} as cation (Table 3) This is in contrast to the activity of adenine phosphoribosyltransferase which showed highest activity in K phosphate buffer with Mn^{++} as cation (12) Attempts to demonstrate the optimal conditions for the enzyme activities with hypoxanthine, guanine and xanthine as substrates have not been considered an issue of this paper

Requirements for Mn^{++} and PRPP The purine phosphoribosyltransferases have an absolute requirement for cations and PRPP showing no activities after dialysis for 24 hours without Mn^{++} or without PRPP (Table 4 expt. no. 1)

Stability of the enzymes. If hypoxanthine was the substrate, the transferase activity would only be about 40 per cent after 24 hours of dialysis whereas there was no loss of activity if guanine and xanthine were used as substrates (Table 4) This difference is less pronounced after freezing of the extracts for 11 to 18 days at −20 C. The loss of activity with hypoxanthine is about 14 per cent only (Table 4)

If extracts of N meningitidis were treated at 45 and 50 C, the phosphoribosyltransferase activities toward guanine and xanthine decreased at the same rate whereas the activity toward hypoxanthine decreased more rapidly (Fig 2 and 3) Heating the extract for 40 minutes at 50 C destroyed all activities (Fig 3) as did heating for 5 minutes at 60 C (not shown)

Effect of parachloromercuribenzoate Contrary to the findings by adenine phosphoribosyltransferase (12) the enzyme activities with hypoxanthine, guanine and xanthine as

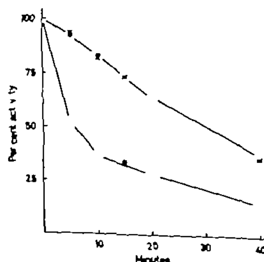


Fig 2 Rate of heat inactivation of purine phosphoribosyltransferase activities at 45 C in an extract from *N meningitidis*. Portions of 0.6 ml extract prewarmed to 37 C, were placed in water bath for the times shown, chilled in an ice-water bath and examined for activity The assay was performed as described under Methods with Tris/HCl buffer and Mn^{++} as cation. 10 µl extract contained 4.6 µg protein. Using hypoxanthine (●——●) guanine (○——○) and xanthine (×——×) as phosphoribosyl acceptors, the respective activities of the zero time samples were 2.29 1.90 and 2.36 nanomoles purine base converted in 10 minutes.

TABLE 4 *Purine Phosphoribosyltransferase Activities in Extracts from N meningitidis*

Expt. No.	State of extract	Protein µg/assay	Cation added	Substrate added	^{14}C-purine base converted nanomoles			Per cent activity		
					Hx	G	X	Hx	G	X
1	New	3.2	Mn^{++}	PRPP	1.72	1.28	1.52	100	100	100
	Dialysed 24 hours*	3.2	Mn^{++}	PRPP	0.75	1.25	1.53	44	98	101
	24 hours	3.2	None	PRPP	0	0	0	0	0	0
	24 hours*	3.2	Mn^{++}	None	0	0	0	0	0	0
	Kept frozen at 20° C for 18 days	3.2	Mn^{++}	PRPP	1.49	1.37	1.45	87	107	94
2	New	5.8	Mn^{++}	PRPP	3.60	2.60	2.90	100	100	100
	Dialysed 24 hours	5.8	Mn	PRPP	1.32	2.76	2.82	37	106	97
	Kept frozen t −20° C for 11 days	5.8	Mn	PRPP	3.22	2.81	2.78	89	108	96
3	New	3.1	Mn^{++}	PRPP	1.53	1.20	1.49	100	100	100
	Kept frozen at 20 C for 14 days	3.1	Mn^{++}	PRPP	1.26	1.30	1.42	82	108	95

Experimental procedure as described under Methods with Tris/HCl buffer Extract dialysed against 100 volumes of Tris/HCl buffer 0.05 M pH 7.4 t 4 C.

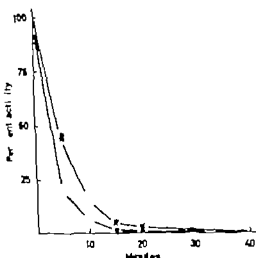


Fig 3 Rate of heat inactivation of purine phosphoribosyltransferase activities at 50 C in an extract from *N meningitidis* Assay as described in the legend to Fig. 2. The extract contained 4 µg protein in 10 µl. With hypoxanthine (●——●) guanine (○——○) and xanthine (×——×) as phosphoribosyl acceptors, the respective activities of the zero time samples were 1 75 1.68 and 2.02 nanomoles purine base converted in 10 minutes.

substrates were very sensitive to the sulphhydryl blocker parachloromercuribenzoate (PCMB) in the same concentration of 0.1 mM (Table 5) No activities were left if the extract was shaken with PCMB for one minute at 37° C before the addition of PRPP (Table 5 expt. no. 1) If the reaction was initiated by the addition of extract, the loss of activity with hypoxanthine and guanine was on an average 75 and 72 per cent, whereas the loss of activity with xanthine was 91 per cent. In experiments using adenine under similar experimental conditions, no inhibition was obtained with PCMB (12)

Estimation of amount of PRPP The transferase reaction if hypoxanthine, guanine and xanthine were used as substrates seems to go to completion in the direction of IMP GMP and XMP respectively when a limited amount of purine base or PRPP is present (Table 6 Fig. 4) Maximal initial velocity of hypoxanthine phosphoribosyltransferase in meningococcal extract was found in Tris/HCl buffer and with Mn^{++} as cation (Fig. 4)

Guanylate Kinase

This enzyme was easily demonstrated in meningococcal extract (Fig. 5) Nearly all the ^{14}C-GMP was converted into radioactive GDP and GTP within 5 minutes in the presence of ATP and Mg^{++} After 5 minutes there was a gradual interconversion of the nucleotides.

DISCUSSION

The radioactive purine bases hypoxanthine, guanine and xanthine are like adenine (12) readily taken up by dividing cells of *N meningitidis* Both adenine and hypoxanthine are incorporated into the adenosine and gua

TABLE 5. *Effect of Parachloromercuribenzoate on Purine Phosphoribosyltransferase Activities in Crude Extract from* N meningitidis

Expt. No.	Protein µg/assay	Chemical added final conc. 0.1 mM	^{14}C-purine base converted nanomoles Hx	G	X	Per cent activity Hx	G	X
1	3.1	None	1.53	1.20	1 49	100	100	100
	3 1	PCMB*	0.36	0.24	0.13	23	20	8.7
	3 1	PCMB**	0	0	0	0	0	0
2	3.8	None	3.60	2.60	2.90	100	100	100
	3 8	PCMB*	0.92	0.93	0.25	25	36	8.6

Experimental conditions as described under Method with Tris/HCl buffer and Mn^{++} as cation. Reaction initiated by the addition of extract. **Reaction mixture shaken in the water bath at 37 C for one minute before the reaction was started with PRPP PCMB was dissolved in KOH and neutralized by HCl.

TABLE 6 *Purine Phosphoribosyltransferase Activities in Cell-free Undiluted Extract from N. meningitidis*

Cation added	Conc. of cation mM	^{14}C-purine base added 5 nanomoles	^{14}C-purine base converted nanomoles
Mn^{++}	1	Hx	5.0
Mn^{++}	1	G	5.2
Mn^{++}	1	X	5.1
Mg^{++}	10	Hx	5.3
Mg^{++}	10	G	5.1
Mg^{++}	10	X	5.2

Experimental conditions as those described under Methods with Tris/HCl buffer 10 µl extract contained 70 µg protein.

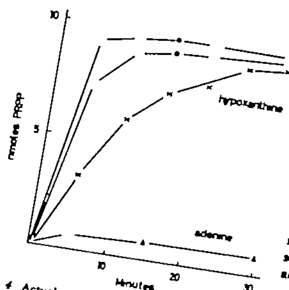


Fig 4 Actual concentration of PRPP in solution determined by hypoxanthine phosphoribosyltransferase in crude undiluted extract from *N meningitidis* Procedure as described under Methods. The extract had been kept frozen at –20 C for 8 days. 10 µl extract contained 70 µg protein. (●——●) Tris/HCl buffer 1 mM Mn^{++} (○——○) Tris/HCl buffer 10 mM Mg^{++} (×——×) K-phosphate buffer 10 mM Mg^{++} (▲——▲) K-phosphate buffer 1 mM Mn^{++} When adenine was the substrate 0.05 µmole ^{14}C-8-adenine (specific activity 9.9 µCi/µmole) was used.

nosine 5 nucleotides as might be expected according to our knowledge of their intermediary metabolism (Fig 1) (22) Contrary to this, guanine and xanthine are utilized for incorporation into the guanosine 5 nucleotides only in *N meningitidis* In *E coli* and *S typhimurium* exogenous guanine and xanthine are incorporated into the adenosine 5 nucleotides as well (22) The failure of *N meningitidis* to do so shows that this microbe lacks a functional GMP reductase. This fact has been demonstrated (*S Jyssum & K. Jyssum* to be published) The yeast *Torulopsis utilis* and *Corynebacterium diphtheriae* also seem to lack GMP reductase (22)

The results clearly show that *N meningitidis* contains enzymes by which to convert hypoxanthine guanine and xanthine to their monophosphates and that these activities seem to be distinct from the activity toward adenine this last enzyme not being sensitive

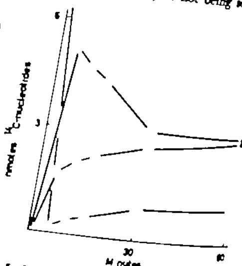


Fig 5 Variation of the radioactivity of GMP (×——×) GDP (●——●) and GTP (○——○) during incubation of undiluted meningococcal extract with ^{14}C-GMP ATP and Mg^{++} (see Methods) 25 µl extract with 0.2 mg protein was used in the assay

to inhibition by PCMB (12) and the activity being negligible in diluted meningococcal extracts (Table 2)

Earlier attempts to separate the hypoxanthine-guanine complex in bacterial extracts were unsuccessful (1 4 15) *Krenitsky et al.* (18) re-investigated the problem, and their results, based on chromatographic resolution and heat inactivation patterns, indicated the existence of at least two different enzymes in *E. coli* preparations. One of these was strongly active if hypoxanthine was used as substrate and only weakly active if guanine was used as substrate. The other enzyme worked primarily with either guanine or xanthine as substrate and had only slight activity for hypoxanthine. Genetic separation of hypoxanthine and guanine-xanthine phosphoribosyltransferase activities has been obtained by mutations in *S typhimurium* by *Gots et al.* (8) and *Chou & Martin* (5) Also in the present case the simplest model which is most consistent with the data is that *S typhimurium* contains two guanine hypoxanthine phosphoribosyltransferases with the specificities outlined for the *E coli* enzymes. The heat inactivation patterns with crude extracts from *N meningitidis* and also the difference in the enzyme activities in dialysed and frozen extracts, are consistent with the presence of one enzyme that is strongly active with hypoxanthine and another that is strongly active with guanine and xanthine as substrates.

The enzyme activities, using guanine and hypoxanthine as substrates, were found to be less susceptible to inhibition by PCMB than the activity to be observed if xanthine was used as substrate (Table 3) Whether this points to an activity of hypoxanthine phosphoribosyltransferase with guanine as substrate remains to be seen. Both the hypoxanthine and the guanine phosphoribosyltransferases are reported to be sensitive to the action of PCMB in other systems (19 24) whereas the activity of xanthine phosphoribosyltransferase from *S faecalis* was not affected by this chemical (23)

The hypoxanthine as well as the guanine xanthine phosphoribosyltransferase reaction seems to be irreversible and to go to completion in meningococcal extracts. Incubation of the hypoxanthine reaction mixture (Fig 4) for another 15 minutes did not diminish the amount of IMP formed, and incubation of 25 μl undiluted meningococcal extract with ^{14}C-8-GMP and $MgCl_2$ for 60 minutes in Tris/HCl buffer pH 7 4 showed that the guanosine spot remained unlabelled and that the guanine spot contained only about 7 per cent of the radioactivity originally added to the reaction mixture.

The amount of endogenous PRPP in the experiment (using Hx as substrate) shown in Fig 4 was 0.3 nanomole. This shows that *N meningitidis* is able to synthetize this metabolite. Adenine phosphoribosyltransferase was far less active than the hypoxanthine and guanine-xanthine transferases in the same meningococcal extracts (Table 2, Fig 4) The phosphoribosyltransferases seem to be localized on the bacterial membrane (9) The lower activity of the adenine catalyzed enzyme might then be due to the fact that the solubility of this enzyme is less than that of the other enzymes in the ultrasonic disintegration technique (11) Or more likely the low activity of adenine phosphoribosyltransferase might be accounted for by its instability *Hori & Henderson* (10) and *Nara et al.* (25) have previously reported that this enzyme is unstable.

The extract from *N meningitidis* had pronounced activities corresponding to guanylate and guanosinediphosphate kinases (Fig. 5) Since GMP cannot support growth in combination with adenine of the adenine-guanine mutant (12) it may be assumed, as in the case of AMP (12) that intact GMP is not taken up by *N meningitidis* cells and that outside the cell GMP is not broken down to guanine in amounts sufficient to support growth.

REFERENCES

1 *Adye J C & Gots J S* Further studies on genetically altered purine nucleotide pyrophos-

phosphorylases of *Salmonella*. Biochim. biophys. Acta (Amst.) *118* 344–350 1966.

2. *Berlin R D & Stadtman E. R.* A possible role of purine nucleotide pyrophosphorylases in the regulation of purine uptake by *Bacillus subtilis*. J biol. Chem. *241:* 2679–2686 1966

3 *Brockman R W., Debavadi, C S., Stutts P & Hutchison D J* Purine ribonucleotide pyrophosphorylases and resistance to purine analogues in *Streptococcus faecalis*. J biol. Chem. *236* 1471–1479 1961

4 *Carter C E.:* Reaction of 6-mercaptopurine with inosine- and guanosine 5'-phosphate pyrophosphorylase purified from *E. coli*. Biochem. Pharmacol. *2* 105–111 1959

5. *Chou J Y & Martin, R. G* Purine phosphoribosyltransferases of *Salmonella typhimurium*. J Bact. *112* 1010–1013 1972

6. *Commission on Biochemical Nomenclature* Enzyme nomenclature, American Elsevier Publishing Company Inc. New York, 1973

7 *Davis, B D & Mingioli E. S.* Mutants of *Escherichia coli* requiring methionine or vitamin B_{12}. J Bact. *60* 17–28, 1950

8. *Gots J S Benson C E. & Shumas S R.* Genetic separation of hypoxanthine and guanine-xanthine phosphoribosyltransferase activities by deletion mutations in *Salmonella typhimurium*. J Bact. *112* 910–916, 1972.

9 *Hochstadt-Ozer J & Stadtman E. R.* The regulation of purine utilization in bacteria II Adenine phosphoribosyltransferase in isolated membrane preparations and its role in transport of adenine across the membrane. J biol. Chem. *246* 5304–5311 1971

10. *Hori M & Henderson J F* Purification and properties of adenylate pyrophosphorylase from Ehrlich ascites tumor cells. J biol. Chem. *241* 1406–1411 1966

11 *Jyssum S* Utilization of thymine, thymidine and TMP by *Neisseria meningitidis* 2. Lack of enzymes for specific incorporation of exogenous thymine, thymidine and TMP into DNA. Acta path. microbiol. scand. Sect. B, *79* 778–788, 1971

Jyssum S Purine metabolism in *Neisseria meningitidis* 1 Utilization of exogenous adenine. Acta path. microbiol. scand. Sect. B, *82* 508–520 1974

13 *Jyssum S & Jyssum K* Utilization of thymine thymidine and TMP by *Neisseria meningitidis* 1 Growth response and uptake of labelled material. Acta path. microbiol scand. Sect. B, *78* 683–691 1970

14 *Kalle G P & Gots J S* Xanthylic pyrophosphorylase of *Salmonella typhimurium*. Fed. Proc *20* 358 1961

15 *Kalle G P & Gots J S* Alterations in purine nucleotide pyrophosphorylases and resistance to purine analogues. Biochim. biophys. Acta (Amst.) *53* 166–173 1961

16 *Krebs H A & Hems R.* Some reactions of adenosine and inosine phosphates in animal tissues. Biochim. biophys. Acta (Amst.) *12* 172–180 1953

17 *Krenitsky T A., Neil, S M., Elion G. B & Hitchings G H* Adenine phosphoribosyltransferase from monkey liver Specificity and properties. J biol. Chem. *244* 4779–4784, 1969

18. *Krenitsky T A., Neil S M & Miller R. L.* Guanine and xanthine phosphoribosyltransfer activities of *Lactobacillus casei* and *Escherichia coli*. Their relationship to hypoxanthine and adenine phosphoribosyltransfer activities. J biol. Chem. *245* 2605–2611 1970.

19 *Krenitsky T A & Papaioannou R.:* Human hypoxanthine phosphoribosyltransferase II. Kinetics and chemical modification. J biol Chem. *244* 1271–1277 1969

20 *Krenitsky T A Papaioannou, R. & Elion, G B.* Human hypoxanthine phosphoribosyltransferase I Purification, properties and specificity J biol Chem. *244* 1263–1270, 1969.

21 *Lowry O H Rosebrough N J Farr A. L & Randall, R J* Protein measurement with the Folin phenol reagent. J biol. Chem. *193* 265–275 1951

22. *Magasanik B* Biosynthesis of purine and pyrimidine nucleotides. In Gunsalus, I. C. & Stanier R. Y (Ed.) The Bacteria, Vol. III Academic Press, New York 1962, p 295–334

23 *Miller R L Adamczyk, D L., Fyfe J A & Elion G B.* Xanthine phosphoribosyltransferase from *Streptococcus faecalis*. Properties and specificity Arch. Biochem. *165* 349–358, 1974

24 *Miller R L Ramsey G A., Krenitsky T A & Elion G B* Guanine phosphoribosyltransferase from *Escherichia coli* specificity and properties Biochemistry *11* 4723–4731 1972.

25 *N e T Misawa, M Komuro T & Kinoshita S* Production of nucleic acid-related substances by fermentative processes. Part XXI Biosynthetic mechanisms involved in 5'-purine ribonucleotide production by *Brevibacterium ammoniagenes* Agr biol. Chem. *33* 358–369 1969

26 *Rebrova T T & Debov S S* Isolation and partial purification of purine nucleotide pyrophosphorylases from *Brevibacterium ammoniagenes* ATCC No. 6872. Vop. med. Khim. *17* 625–631 1971

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PURINE METABOLISM IN *NEISSERIA MENINGITIDIS*

4. *Interconversion of Purine Ribonucleotides*

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Radioactive hypoxanthine and adenine were readily incorporated into the nucleic acids (RNA + DNA) of the prototrophic strain M1 of *Neisseria meningitidis* with labelling of the purine bases adenine and guanine. Contrary to this, adenine was incorporated into the nucleic acids with labelling of adenine only in the purine mutant 6-1c which requires adenine and guanine for growth. Guanine was in both strains incorporated into the nucleic acids with labelling of the purine base guanine only. Crude meningococcal extracts from the prototrophic strain M1 and strain 6-1b requiring histidine, proline, adenine and guanine for growth (*his pro A G*) were found to have activities corresponding to adenylosuccinate synthetase (EC 6.3.4.4), adenylosuccinate lyase (EC 4.3.2.2.), inosine 5'-phosphate dehydrogenase (EC 1.2.1.14)) and guanosine 5'-phosphate synthetase (EC 6.3.5.2). This last enzyme was active also with $(NH_4)_2SO_4$ as amino donor. No activity corresponding to guanosine 5'-phosphate reductase (EC 1.6.6.8) could be demonstrated in either strain. The findings show that the mutant strains 6-1b and 6-1c are both blocked between aminoimidazolecarboxamide ribonucleotide (AICA-R) and inosine 5'-phosphate (IMP). Transformation experiments with mutants requiring adenine and guanine for growth indicate that the negative property of these mutants is due to a lesion in one genetic locus or at least in loci that are very closely linked.

Key words: *Neisseria meningitidis*; purine metabolism; purine ribonucleotides; interconversion.

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In an earlier report it was shown that the purine mutant 6-1b (*his pro A G*) of *N. meningitidis* could grow with xanthine instead of guanine and hypoxanthine could be utilized as the only purine base (16). Crude extracts from *N. meningitidis* had activities corresponding to an adenine phosphoribosyltransferase (16), a hypoxanthine phosphoribosyltransferase (18) and a phosphoribosyltransferase which seemed specific for guanine and xanthine as substrates (18). The phosphoribosyltransferases convert the purine bases to their respective monophosphates by condensation with 5'-phosphoribose 1-diphosphate (PRPP).

In the present work, the labelling of the nucleic acids bases (purine and pyrimidine)

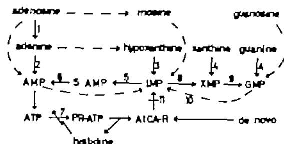


Fig 1 Purine metabolism in *Neisseria meningitidis*. *1*–adenosine nucleosidase *2*–adenine phosphoribosyltransferase *3*–hypoxanthine phosphoribosyltransferase *4*–guanine-xanthine phosphoribosyltransferase *5*–adenylosuccinate synthetase; *6*–adenylosuccinate lyase *7*–ATP phosphoribosyltransferase *8*–IMP dehydrogenase *9*–GMP synthetase *10*–GMP reductase *11*–The adenine, guanine mutants blocked in the conversion of AICA R to IMP Broken lines indicate reactions which do not occur. PR-ATP phosphoribosyl-ATP

from radioactive adenine, hypoxanthine and guanine of the prototrophic strain M1 and of the adenine, guanine mutant 6-1c (*A G*) of *N meningitidis* has been examined.

Fig 1 shows the interconversion of the purine ribonucleotides known to occur in the enterobacteria *Escherichia coli* and *Salmonella typhimurium* (25) The following enzyme reactions were measured in crude extracts from strain M1 and 6-1b (*his pro A G*) of *N meningitidis* (see Fig 1) *5* – adenylosuccinate synthetase (EC 6.3 4 4) and *6* – adenylosuccinate lyase (EC 4.3.2.2) which, measured in the same experiment, catalyze the following reactions IMP + GTP + L aspartate = S-AMP + GDP + orthophosphate and S-AMP = AMP + fumarate respectively *8* – IMP dehydrogenase (EC 1 2.1 14) IMP + NAD + H_2O = XMP + NADH *9* – GMP synthetase (EC 6.3.5.2) XMP + L-glutamine + ATP + H_2O = GMP + L-glutamate + AMP + pyrophosphate and *10* – GMP reductase (EC 1.6.6.8) GMP + NADPH = IMP + $NADP^+$ + ammonia. This last enzyme was also measured with extracts from *E. coli* for control.

Subsequently transformation experiments with an adenine, guanine mutant have been performed to elucidate the nature of the genetic block in this mutant.

Abbreviations AMP adenosine 5' phosphate ATP adenosine 5'-triphosphate S-AMP adenylosuccinate GMP guanosine 5'-phosphate GDP guanosine 5'-diphosphate GTP guanosine 5'-triphosphate XMP xanthosine 5'-phosphate NAD nicotinamide adenine dinucleotide and NADH its reduced form $NADP^+$ nicotinamide adenine dinucleotide phosphate and NADPH its reduced form.

MATERIALS AND METHODS

Strains The meningococci were the wild-type strain M1 of group B and the following mutants from the wild type strain 6-1b (*his pro A G cp*) 8-1b (*his arg A G cp*) 6-1c (*A G cp*) The adenine, guanine mutants (*A G*) were isolated by a selective procedure with methotrexate from the mutants M1 (*his pro*) and M1 (*his arg*) (19) The mutant 8-1b (*his arg A G cp*) was used as recipient during transformation. The mutant 6-1c (*A G cp*) a spontaneous back mutant from 6-1b, as isolated by heavy inoculates of 6-1b in heart infusion broth (HIB) on Medium A plates (8) supplemented with adenine and guanine and grown for at least 4 days as previously described (8) The streptomycin resistant mutant of strain M1 used for the production of transforming DNA (4) was the same as that previously used (9) The genetically competent variant (*cp*) and the genetically incompetent variant (*p*) were controlled as previously described (14) A strain of *E. coli* K12 was used for comparison in one experiment.

Media. Blood agar plates or basal medium (Medium A) plates were used as solid media (8). The basal medium plates were supplemented with the growth factors required (22) Fluid medium was the enriched, defined Medium KC (19)

Growth conditions N meningitidis was grown as previously described (8, 19)

Cell free extracts. Overnight cultures on blood agar plates were harvested and treated as before (15) The buffer used for suspending the cells (appr 0 1 g wet cells per ml buffer) was made 2 mM with dithiothreitol (DTT) or reduced glutathione (GSH) (stated in the actual experiment) before disintegration of the cells. The extracts were used immediately if not otherwise stated Protein was estimated by the procedure of *Lowry et al.* (24)

Labelling of nucleic acids. Cells harvested from overnight blood agar plates were suspended in 15 ml of Medium KC (19) and grown for 4 to 5 generations with the radioactive labelled purines The wild-type strain M1 was supplemented with ^{14}C-8-adenine ^{14}C-8- 1 7 µg per ml culture of ^{14}C-8-adenine guanine or ^{14}C-8-hypoxanthine. The mutant strain 6-1c (*A G*) was supplemented as follows *1* 7.2 µg ^{14}C-8-adenine, 7.2 µg unlabelled guanine and 0.1

TABLE 1 *Reference Values of Standards Used for Identification of Radioactive Metabolites on Cellulose Thin Layer (20 × 20 cm)*

Internal standard	NH_4-acetate 1M-pH 7.0	Isobutyric acid phase*	n-Butanol phase§
Hypoxanthine	0.55	0.65	0.59
Guanine	0.34		0.53
Inosine	0.76		0.55
Guanosine	0.62		0.47
IMP	0.85	0.50	0.24
GMP	0.75		0.19
XMP		0.19	0.17
S-AMP		0.29	0.16
AMP		0.61	0.32
ADP		0.49	0.17
ATP		0.39	0.11

* Isobutyric acid phase: isobutyric acid – conc. NH_3 – H_2O – 0.1 M EDTA (500 21 279 8, by vol.)
§ n-Butanol phase n-butanol – acetone – acetic acid – conc. NH_3 – H_2O (45 15 10 2 28, by vol.) (15)

µg unlabelled hypoxanthine per ml culture 2-7.2 µg ^{14}C-8-guanine, 7.2 µg unlabelled adenine and 0.8 µg unlabelled hypoxanthine per ml culture 3-7.5 µg ^{14}C-8-hypoxanthine per ml culture. The bases were adjusted to the following specific activities with the unlabelled compound adenine 0.07 µCi/µg; guanine 0.06 µCi/µg and hypoxanthine 0.02 µCi/µg.

Preparation of ^{14}C-labelled nucleic acids (N.A.) About 30 mg, wet weight, of ^{14}C-labelled cells washed three times with ice cold KC Medium, were treated according to *Be re* (4) the procedure scaled down according to the amount of bacteria. The supernatant with the nucleic acids was over layered with 2 volumes of 96 per cent ethanol and kept at 4 C overnight whereby precipitation of the nucleic acids was achieved. After centrifugation the N.A. pellet was washed in 70 per cent ethanol and dried.

Radioactive base analysis. Base hydrolysis by $HClO_4$ was performed according to *Bendich* (2) The pellet of N.A. was mixed with 10 µl 70 per cent $HClO_4$ in a glass-stoppered test tube and heated at 100° C for 60 minutes. The mixture was cooled and 40 µl water was added. After centrifugation, 5 µl of the supernatant was spotted on cellulose thin layer (20 × 20 cm) as before (16) The chromatogram was developed in 2 N HCl in isopropanol-water (65 35) (2) and for control in n-butanol- 0.1 N NH_3 (6 1) (2) for 17 cm. Exposure to X-ray film and counting of labelled spots were as in previous procedures (16)

Adenylosuccinate synthetase EC 6.3.4.4 (5) and *adenylosuccinate lyase* EC 4.3.2.2 (5) Enzyme activities were assayed by mixing 5 µmoles K-phosphate buffer pH 7.8 0.05 µmole GSH (neutralized with KOH) 0.5 µmole $MgCl_2$; 0.3 µmole *PRPP* 8.5 nanomoles ^{14}C-8-hypoxanthine (59 µCi/µmole) and 10 µl crude extract in 0.05 M K-phosphate buffer pH 7.8 made 2 mM with GSH. This reaction mixture of 50 µl was shaken in water bath at 37 C for 30 minutes. During this time, all the ^{14}C-hypoxanthine was transformed to ^{14}C-IMP (16) To the reaction mixture, 0.5 µmole L-aspartic acid and 0.5 µmole GTP (both neutralized with KOH) was added to total of 60 µl. The reaction was run for additional 5 10 15 30 and 60 minutes. Protein was precipitated with 60 µl 96 per cent ethanol at 0° C followed by 5 µl 0.1 M potassium EDTA. 5 µl of the supernatant was subjected to two-dimensional chromatography on cellulose thin layer (20 × 20 cm) in the isobutyric acid solvent (Table 1) in the first direction and in the n-butanol solvent system (Table 1) in the second direction. The chromatogram was run twice in the last solvent to achieve good separation of the spots. Radioautography and counting was as before (16)

IMP dehydrogenase EC 1.2.1.14 (5) The reaction mixture for the production of ^{14}C-IMP was as outlined above, except that 0.05 µmole $MnCl_2$ was used instead of Mg^{++} since this last ion is *reported* to be inhibitory to the *E. coli* IMP dehydrogenase activity (30) After 30 minutes 0.1 µmole of NAD was added. The rest of the procedure was as mentioned above except that *chromatography was in* the isobutyric acid solvent only in one dimension for 17 cm (Table 1) The chromatogram was run twice in this solvent. The rest of the procedure was as above.

GMP synthetase EC 6.3.5.2 (5) was assayed according to *Magasanik* (26) with small alterations, by mixing in a total volume of 0.25 ml 40 µmoles Tris/HCl buffer pH 7.6 1 µmole ATP 4 µmoles

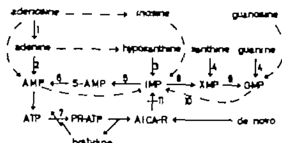


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TABLE 3 *Activities of Adenylosuccinate Synthetase and Adenylosuccinate Lyase in Crude Extracts from N meningitidis*

Strain	Protein mg/assay	Time of incubation min	IMP not converted nmoles	S-AMP formed nmoles	Nucleotides formed nmoles AMP + ADP + ATP
M1	0.06	5	7.85	0.32	0.14
wild-type	0.06	10	7.38	0 46	0 48
	0.06	15	7.08	0.47	0.85
	0.06	30	6.54	0 42	1.81
	0.06	60	3.39	0.32	3 16
	0.06	60*	7.81	0 10	0.28
6-1b	0.05	5	8.07	0.34	0 19
(*his pro A G*)	0.05	10	6.94	0.34	0.52
	0.05	15	6.60	0 42	0.84
	0.05	30	6.59	0.28	1 79
	0 05	60	5 42	0.19	3.21
	0.05	60*	8.23	0.03	0.19

Experimental conditions as described under Methods.
* Control, GTP not added. In these experiments all the ^{14}C-8-hypoxanthine was converted to IMP in 30 minutes (see Methods)

TABLE 4 *Activities of IMP Dehydrogenase in Crude Extracts from N meningitidis*

Strain	Protein mg/assay	Time of incubation min	Hypoxanthine not converted nmoles	IMP not converted nmoles	XMP formed nmoles
M1	0 06	5	1.20	7.31	0.06
wild-type	0.06	10	0.63	7 76	0.14
	0.06	15	0 54	7.96	0.20
	0.06	30	0.24	7.80	0.30
	0.06	60	0 19	7.88	0.46
	0.06	60*	0.23	8.06	0.08
6-1b	0.05	5	0.85	7.37	0.08
(*his pro A G*)	0.05	10	0.48	8.07	0 14
	0.05	15	0.35	8.23	0.19
	0.05	30	0 19	8.21	0.27
	0.05	60	0.10	8 36	0.38
	0.05	60*	0.09	8.78	0.06

Experimental conditions as described under Methods.
* Control, NAD^+ not added.

Enzyme Measurements

Adenylosuccinate synthetase and adenylosuccinate lyase Table 3 shows that extracts from both the wild-type strain M1 and the mutant strain 6-1b (*his pro A G*) have enzyme activities for conversion of IMP to S-AMP and for conversion of S-AMP to AMP When GTP was omitted, the crude extracts still showed some enzyme activities which must be due to some GTP present in these extracts. The enzyme activities were measured in K-phosphate buffer since Tris

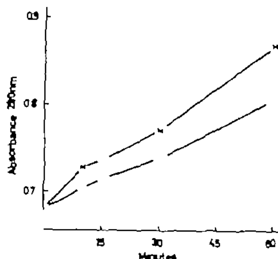


Fig 2 Activities corresponding to GMP synthetase *in crude meningococcal extract from strain M1* with glutamine (×——×) and with $(NH_4)_2SO_4$ (●——●) as substrate. Experimental conditions as described under Methods. 50 µl extract which had been kept frozen overnight at −20 C contained 0.3 mg protein. Corrections were made for endogenous changes of absorbancy (samples without XMP)

buffer is reported to be inhibitory to the *synthesis of S-AMP with purified enzyme* from *E coli* (23)

IMP dehydrogenase Table 4 shows the enzyme activity in crude extracts from strain M1 and 6-1b (*his pro A G*) If NAD^+ was omitted from the reaction mixture, some enzyme activity would still be left. K-phosphate buffer was used since IMP dehydrogenase activity is dependent on the presence of monovalent cations (30, 31) and the activity in this buffer at pH 7.55 was reported to be near maximum with purified enzyme from *Bacillus subtilis* (31)

GMP synthetase Fig 2 and 3 show the enzyme activities with L-glutamine and $(NH_4)_2SO$ as substrates in crude extracts from strain *M1* and strain *6-1b* (*his pro A G*) In several experiments the enzymes from both sources showed the same preference *for glutamine (32 mM) over* ammonia (32, 80 160 and 320 mM $(NH\ )_2SO$) as amino donor With extracts from both strains, the amount of GMP formed in 60 minutes was about 68 per cent with $(NH_4)_2SO_4$ (160 mM) *compared with the* amount formed with glutamine as substrate (Fig 2 and 3)

GMP reductase All attempts to demonstrate an active GMP reductase in meningococcal extracts (strain 6-1b (*his pro A G*) and strain M1) were negative. Extracts obtained from sonically treated cells have previously been found to have NADPH oxidizing activity (11) Therefore, in some experiments, 0.5 µmole glukose-6-phosphate (G-6-P) was added to the reaction mixture to ensure the regeneration of NADPH by means of G-6-P dehydrogenase present in the crude extracts (10) but no GMP reductase activity could be found. In contrast, *E coli* extracts always showed activity with incorporation of ¹⁴C-GMP into IMP even without G-6-P added (Fig 4)

Transformation experiments. In the transformation experiments each of the nutritional markers of the mutant 8-1b (*his arg A G cp*) *was analysed with regard to linkage.* Table 5 shows that the markers *A* and *G* were 100 per cent linked in reciprocal crosses, i.e. 100

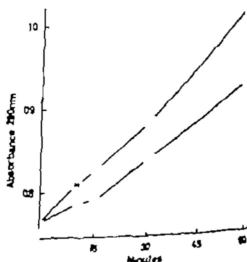


Fig 3 Activities corresponding to GMP synthetase in crude meningococcal extract from strain 6-1b (*h pro A G*) with glutamine (×——×) and with $(NH\ )_2SO$ (●——●) as substrate. Experimental conditions as described under Methods. 50 µl extract containing 0.28 mg protein. Corrections were made for endogenous changes of absorbancy (samples without XMP)

with differential substrate preferences and that, upon chromatographic purification, a synthetase was obtained showing no activity with glutamine. However genetic analysis of *E. coli* K12 seems to point to the existence of only one gene for an enzyme which catalyzes amination of XMP (29) In a recent report, *Lee* & *Hartman* (21) conclude that *E coli* B possesses a single enzyme responsible for conversion of XMP to GMP The enzyme preferentially used glutamine as amino donor under the specified conditions of isolation and assay Partially purified GMP synthetase from *B subtilis* is also reported to utilize glutamine more effectively than $(NH_4)_2SO_4$ as the amino donor (6) The findings obtained by the enzyme from *N meningitidis* (Fig 2 and 3) are then analogous to those observed in *B subtilis* and in *E. coli* B by *Lee* & *Hartman* (21)

Whole cells of the wild type strain M1 of *N meningitidis* are capable of forming both AMP and GMP from exogenous radioactive adenine (16) The enzymes which convert ATP to IMP must therefore be present in this microbe (Fig 1) *N meningitidis* is capable of forming both AMP and GMP by synthesis *de novo* as shown by the fact that the microbe can be adapted to growth on synthetic media containing glucose as the only source of carbon and ammonia as the only source of nitrogen (7)

N meningitidis seems not to contain an active AMP deaminase with formation of IMP since adenine alone could not support growth of the adenine, guanine mutant 6-1b (*his pro A G*) Any attempts to demonstrate such an activity in crude meningococcal extracts with ^{14}C-8-AMP were negative (not shown)

The purine metabolism in *N meningitidis* (16, 17 18) must then be assumed to be as shown in Fig 1 No attempts were made in the present work to study the mechanisms of conversion of purine ribonucleotides to deoxyribonucleotides.

The finding of a 100 per cent linkage, in the transformation experiments, between the nutritional requirements A and G of the mutant 8-1b (*his arg A G cp*) certainly shows that the two markers are transformed together on the same molecule of transforming DNA. The most likely explanation of such a linkage in transformation is that the two nutritional requirements in fact represent the phenotypic expression of a single genetic lesion. If not, the two lesions must at least be extremely closely located on the chromosome. In other experiments (not shown) many back mutants have been picked up from the auxotroph 6-1b (*his pro A G cp*) All mutants that grew on his + pro + G also grew on his + pro. This adds to the assumption that the double requirement (A+G) represents one genetic lesion

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REFERENCES

1 *Abrams R & Bentley M* Biosynthesis of nucleic acid purines. III Guanosine 5' phosphate formation from xanthosine 5'-phosphate and L-glutamine Arch. Biochem. 79 91–110, 1959

2. *Bendich A* Methods for characterization of nucleic acids by base compositions. In Colowick, S. P & Kaplan, N O. (Ed.) Methods in Enzymology Vol. III Academic Press, New York 1957 p. 715–723.

3 *Brevet A H degaard J & Roche J* Sur la spécificité des donateurs d'azote pour la xanthosine 5'-phosphate aminase d'*Escherichia coli* B, in vitro C. R. Soc. Biol. (Paris) *161* 1938–1944 1967

4 *Bøvre K* Studies on transformation in *Moraxella* and organisms assumed to be related to *Moraxella*. 1 A method for quantitative transformation in *Moraxella* and *Neisseria*, with streptomycin resistance as the genetic marker Acta path. microbiol. scand. *61* 457 473, 1964

5 *Commission on Biochemical Nomenclature* Enzyme nomenclature. American Elsevier Publishing Company Inc. New York, 1973.

6 *Ishii, K & Shiio I* Regulation of purine ribonucleotide synthesis by end product inhibition I Effect of purine nucleotides on inosine 5'-phosphate dehydrogenase xanthosine-5'-phosphate aminase and adenylosuccinate lyase of *Bacillus subtilis*. J Biochem. (Tokyo) *63* 661–669 1968.

7 *Jyssum, K* Assimilation of nitrogen in meningococci grown with the ammonium ion as sole nitrogen source. Acta path. microbiol. scand. 46 320–332, 1959

8. *Jyssum K.* Isolation of auxotrophs from *Neisseria meningitidis*. Acta path. microbiol. scand. 63 435–444 1965.

9 *Jyssum K* Polarity of chromosome replication in *Neisseria meningitidis*. J Bact. 90 1182–1187 1965.

10. *Jyssum K Borchgrevink B. & Jyssum S.:* Glucose catabolism in *Neisseria meningitidis* 1 Glucose oxidation and intermediate reactions of the Embden-Meyerhof pathway Acta path. microbiol. scand. 53: 71–83 1961

11 *Jyssum, K & Jyssum S.*, Glucose catabolism in *Neisseria meningitidis* 2. Reactions of the pentose phosphate pathway and of the Entner Doudoroff route. Acta path. microbiol. scand. 55 437–446, 1962.

12. *Jyssum K Jyssum, S & Gundersen W B.* Sorption of DNA and RNA during transformation of *Neisseria meningitidis*. Acta path. microbiol. scand. Sect. B, 79 363–371 1971

13. *Jyssum K & Lie S* Transformation of auxotrophs of *Neisseria meningitidis*. Acta path microbiol. scand. 63 445–455 1965

14 *Jyssum K. & Lie S* Genetic factors determining competence in transformation of *Neisseria meningitidis* 1 A permanent loss of competence Acta path. microbiol. scand 63 306–316 1965.

15. *Jyssum S* Utilization of thymine, thymidine and TMP by *Neisseria meningitidis* 2. Lack of enzymes for specific incorporation of exogenous thymine, thymidine and TMP into DNA. Acta path. microbiol. scand. Sect. B 79 778–788, 1971

16. *Jyssum, S* Purine metabolism in *Neisseria meningitidis* 1 Utilization of exogenous adenine. Acta path. microbiol. scand. Sect. B, 82 506–520, 1974

17 *Jyssum S* Purine metabolism in *Neisseria meningitidis* 2 Utilization of exogenous adenosine, guanosine and inosine Acta path. microbiol. scand. Sect. B, 82 883–894 1974

18. *Jyssum, S* Purine metabolism in *Neisseria meningitidis* 3 Utilization of exogenous hypoxanthine guanine and xanthine Acta path. microbiol. scand. Sect. B, 83 397–406, 1975

19. *Jyssum S & Jyssum K* Utilization of thymine thymidine and TMP by *Neisseria meningitidis* 1 Growth response and uptake of labelled material. Acta path. microbiol scand. Sect. B 78 683–691 1970

20. *Lagerkvist U* Biosynthesis of guanosine 5 phosphate II Amination of xanthosine 5 phosphate by purified enzyme from pigeon liver J biol. Chem. 233 143–149 1958.

21 *Lee B. H & Hartman S C.*, Preferential utilization of glutamine for amination of xanthosine 5 -phosphate to guanosine 5 -phosphate by purified enzymes from *Escherichia coli*. Biochem. biophys. Res. Commun. 60 918–925 1974

22. *Lie S* Production of auxotrophic mutants of *Neisseria meningitidis* by nitrous acid. Acta path. microbiol. scand. 63 615–622, 1965

23 *Lieberman, I.:* Amination of IMP to AMP-adenylosuccinate synthase (*Escherichia coli*) In. Colowick, S. P & Kaplan, N O (Ed.): Methods in Enzymology Vol. VI. Academic Press, New York 1963 p 100–102.

24 *Lowry O H Rosebrough N J., Farr A L. & Randall, R. J* Protein measurement with the Folin phenol reagent. J biol Chem. 193 265–275 1951

25 *Magasanik B.* Biosynthesis of purine and pyrimidine nucleotides. In Gunsalus, L C. & Stanier R. Y (Ed.): The Bacteria. Vol. III. Academic Press, New York 1962, p. 295–334

26. *Magasanik B* Synthesis and reduction of GMP In Colowick, S. P & Kaplan, N O (Ed.) Methods in Enzymology Vol. VI Academic Press, New York 1963 p. 106–117

27 *Moyed H S & Magasanik, B.* Enzymes essential for the biosynthesis of nucleic acid guanine xanthosine 5 -phosphate aminase of *Aerobacter aerogenes*. J biol. Chem. 226 351–363 1957

28. *Nester E. W & Lederberg, J* Linkage of genetic units of *Bacillus subtilis* in DNA transformation. Proc. Nat. Acad. Sci. (Wash.) 47 52–55 1961

29. *Nijkamp H J J & D Haan P G* Genetic and biochemical studies of the guanosine 5 monophosphate pathway in *Escherichia coli* Biochim. biophys. Acta (Amst.) 145 31–40, 1967

30. *Powell G Rajagopalan K V & Handler P* Purification and properties of inosinic acid dehydrogenase from *Escherichia coli*. J biol. Chem. 244 4793–4797 1969

31 *Wu, T W & Scrimgeour K. G.*, Properties of inosinic acid dehydrogenase from *Bacillus subtilis* II. Kinetic properties. Canad. J Biochem. 51 1391–1398, 1973.

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EXPERIMENTAL EVALUATION OF THE VELVET PAD RINSE TECHNIQUE AS A MICROBIOLOGICAL SAMPLING METHOD

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Raahave, D Experimental evaluation of the elvet pad rinse technique as a microbiological sampling method. Acta path. microbiol. scand. Sect. B, 83 416-424 1975

The use of elvet pads glued to aluminium foil has been evaluated as a microbiological sampling method in quantitative bacterial recovery experiments, and the efficiency and precision have been determined. The velvet pad uptake of *Staphylococcus epidermidis* was 0 93 (or 93 per cent) after sampling from a test surface on blood agar while the release by subsequent imprinting on blood agar was 0 02, the median effective transfer being 0.02. A mechanical rinse and shake procedure of the velvet pad in 0 9 per cent saline followed by centrifugation and surface plating significantly increased the median release to 0 66 the median effective transfer being 0 61 There was no difference in uptake, release and effective transfer between a pure culture of *S epidermidis* and a mixed culture of *S epidermidis* and *Escherichia coli*. Storage of the velvet pad in 0.9 per cent saline for 2 h at room temperature did not influence bacterial recovery significantly in contrast to a significant decrease after storage in saline for 24 h or storage in a dry Petri dish for 2 h. The high and fairly constant efficiency of bacterial recovery of the velvet pad rinse technique suggests that t could be employed clinically

Key words Velvet pad rinse technique microbiological sampling method evaluation.

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Lederberg & Lederberg (1952) introduced velvet into bacteriological laboratory work for transfer of isolates from a plate culture to other selective or indicator agar media. The method has since been known as the replica plating technique, and has been extended to sampling from various colonized human surfaces (*Gorrill & Penikett* 1957 *Saggers & Stewart* 1964 *Holt* 1966 *Reibel et al.* 1967 *Holt* 1971 *Raahave* 1974) *Lederberg & Lederberg* (1952) made a crude estimate of the efficiency of transfer from the initial plates spread with measured numbers of *Escherichia coli* cells Approximately 10 to 30 per cent of the initial cells were transferred to the fabric and again an equal proportion of these was found to be deposited on the replica plates. *Elek & Hilson* (1954) estimated the proportion of organisms trans-

ferred by replica plating to be of the order of 1 in 100 using the described density of inocula.

The present aim was to evaluate the *efficacy* and *precision* of bacterial recovery by the velvet pad technique on an experimental basis. The survival of bacteria after sampling and storage has also been investigated in quantitative bacterial recovery experiments.

MATERIAL AND METHODS

Velvet and Cotton Pads

Pieces of pale, boiled-out velvet (20.0 × 45.0 mm) with closeknit pile were backed by aluminium foil with a water and heat resisting contact adhesive UHU® (*UHU Werk, H V M Fischer Buhl/Baden West Germany*) following the description by *Saggers & Stewart* (1964) and *Holt* (1966). The foil was extended at both ends of the velvet pad for about 20 mm, in order that the pad could be handled without contaminating the velvet itself. The velvet pads were packed in Med Plast® sterilizing bags, heat sealed, and autoclaved at 121 C for 30 min (Fig. 1). After use they were cleansed and re-sterilized. Pads of plain cotton were made and treated in the same way.

Culture Medium

A nutrient agar (blood agar base (*Oxoid*) 4 per cent, glucose 0.1 per cent) in demineralised water pH 7.5 after autoclaving, containing 5 per cent defibrinated horse blood and 0.75 per cent yeast extract (*Difco*) was dispensed into plastic Petri dishes (diameter 14 cm). These allowed easy access into the dish with a view to imprinting the velvet pad on the agar surface (Fig. 2).



Fig. 2. The sampling velvet pad is being imprinted on an uninoculated blood agar plate.

Bacterial Cultures

Two strains were used *Staphylococcus epidermidis* CCM 2434 obtained from the Czechoslovak Collection of Microorganisms and an *E. coli*-strain isolated from a septic wound. 18-h serum broth cultures were diluted to 10⁻ for *S. epidermidis* and to 10^{-4} for *E. coli* to give a convenient control colony count with dense but not confluent growth.

Procedures

Experiments A and B (Fig. 3).—Two ml of diluted bacterial suspension of *S. epidermidis* were pipetted on to the surface of blood agar plate (plate I) and distributed uniformly over the entire surface by rocking the plate. Excess liquid was removed by a Pasteur pipette and the surface was allowed to dry for 10 min. A sterile velvet pad, moistened with 0.9 per cent saline, was then applied firmly and evenly for 2–3 sec to the bacterial test surface. The sampling pad was then imprinted on an uninoculated blood agar plate (plate II), care being taken not to slide the pad,

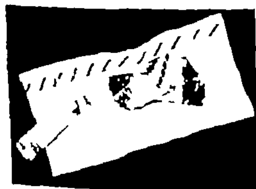


Fig. 1. Sterile velvet pads, 20.0 × 45.0 mm.

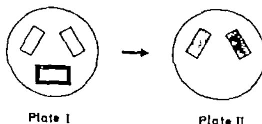


Fig. 3. Experiment B.—After incubation at 37° C for 24 h, control count (window) was made of the original seeded numbers of colony-forming units on plate I. Remaining counts developed on the site of sampling with velvet pads. Transferred counts (hatched) were obtained from plate II.

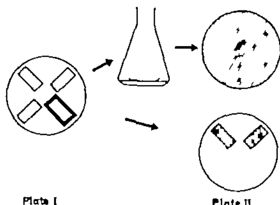


Fig 4 Experiment C.—Bacterial transfer from *plate I to plate II by (1) imprints of velvet and* cotton pads and (2) rinse of the velvet pad followed by centrifugation and surface plating of the precipitate.

in order to avoid smears or tailings of the subsequent colony growth. After the plates had been incubated aerobically *at 37 C for 24 h, a count* was made of the remaining colonies which developed at the site of sampling on plate I and of the colonies which developed after transfer to plate II together with a control count through a 900 mm² template (plate I) expressing the original number of colony forming units. Experiment B devi ted from A in that (1) velvet pad sampling from the test surface was carried out immediately after inoculation and evaporation of visible moisture (30 sec) (2) both new and re-used velvet pads were employed (two impressions on plate *II*) and (3) the overnight broth cultures of *S pidermidis were diluted to 10⁻ by serum instead of by 0.9* per cent saline.

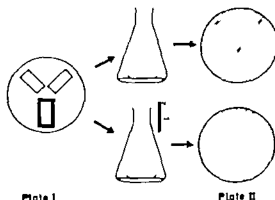


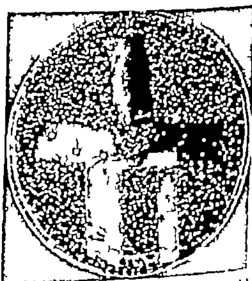
Fig 5 Experiment D —Comparison of bacterial recovery after storage of the velvet pad in saline for 24 h at +4 C and after no storage

Experiment C (Fig 4) —The experiment was similar *to B, but after sampling the test surface,* a further sampling pad was transferred to a 300 ml Erlenmeyer flask with 0.9 *per cent* sterile sodium chloride. The flask with its contents was shaken mechanically for 10 min and the suspension was centrifuged at 3000 rev/min for 30 min. The supernatant was removed, the precipitate resuspended and finally spread over a blood agar plate (plate II) using a bent glass rod. Plain cotton pads were also tested.

Experiments D E and F (Fig 5) —Two ml of a mixed culture of *S epidermidis* (10) and *E. coli* (10⁻⁴) were seeded on to the blood agar surface and after sampling the pads were transferred to Erlenmeyer flasks. Half of the samples followed *the rinse procedure as in experiment C, the other* half was stored at +4 C for 24 h (experiment D) and for 2 h (experiment E) before they ere rinsed, centrifuged and plated.

In experiment F half of the elvet pads were immediately transferred to Erlenmeyer flasks with saline after sampling from the test surface and further prepared as in experiment C, while the others were stored in a sterile dry plastic Petri dish at room temperature for 2 h.

Contamination was examined by including a control velvet pad which was taken through the procedures of all experiments, excepting the contact with the sampling surface.



F g 6 Experiment C (Fig. 4 plate I) —Bacterial test surface of *S pidermidis* after incubation for 24 h at 37 C. Colonies are remaining after sampling with cotton pad (wp) and velvet pad for subsequent imprint (right) and elvet pad to be rinsed (left)

TABLE 1 *Experiment A The Effective Transfer of S. epidermidis by the Velvet Pad Imprint Method*
Experiment B Comparison of New and R -used Velvet Pads

Number of experiments	Colony counts: Plate I Control	Plate I Remaining		Plate II Transferred		Estimation of transfer: Uptake		Release		Effective	
	315	209		5		.34		05		02	
	357	160		11		.53		.06		.03	
	367	126		7		66		.03		.02	
	349	201		14		42		10		04	
	346	174		17		.50		10		.05	
	318	122		4		.62		02		.01	
	311	107		12		.65		06		.04	
l	243	91		6		.63		04		.03	
	299	82		9		72		04		.03	
	294	72		5		75		02		.02	
	286	79		7		72		.03		.02	
	297	110		6		.63		03		.02	
Median											
	313	116		7		.63		04		.03	
l		Re used	New	Re used	New	Re-used	New	Re-used	New	Re-used	New
4	212	15	9	2	2	93	.96	.01	.01	.01	01

Mathematical and Statistical Methods

The *uptake release* and *effective transfer* of bacteria by the velvet pad technique have been calculated from the viable counts expressing the original seeded numbers of colony-forming units on plate I (control counts) the colonies developed after sampling with velvet pad on plate I (remaining counts) and the colonies developed from colony-forming units transferred to plate II (transferred counts) *Uptake* = (control minus remaining)/control *Release* = transferred/(control minus remaining) *Effective transfer* = uptake × release. Relevant comparisons between the experimental results were made by distribution-free statistical tests (*Bradley* 1968)

RESULTS

Experiments A and B.—A protocol for the results is given in Table 1 where the median uptake is 0 63 and the release 0.04 yielding an effective transfer of 0 03 In experiment B, a paired non-parametric statistical analysis (*Wilcoxon*) demonstrated no statistically significant difference between the estimated effective transfers after sampling with new or re-used velvet pads ($p>0.05$) The uptake of both types of velvet pads was high, median 0.93 and 0.96, both differing significantly from the uptake in experiment A ($p<0\,01$ *Mann Whitney*)

Experiment C—The blood agar surface with *S epidermidis* is shown after incubation in Fig 6 with only a few remaining colonies after previous sampling with velvet and cotton pads a template is placed for the control count. The estimated median uptake was 0.97 and 0.93 for cotton and velvet pad, respectively (Table 2) the medians for release being 0.03 and 0 02 and for effective transfer 0.03 and 0 0? showing no statistically significant difference ($p>0\,05$) The few colonies transferred are seen in Fig 7 whereas Fig. 8 shows the colonies recovered by the shake and rinse procedure. By this technique the median release of bacteria was 0 66 and the median effective transfer 0 61 differing significantly from the effective transfer after impression ($p<0\,001$)

Experiment D—The median release of *S epidermidis* was 0.64 and 0 42 (Table 3) and the difference between the effective transfers after storage in saline for 24 h at

TABLE 2. *Experiment C Comparison of the Effective Transfer of S. epidermidis by the Velvet Pad Impression Method and the Velvet Pad Rinse Technique*

Estimation of transfer								
Uptake			Release			Effective		
CPI	VPI	VPR	CPI	VPI	VPR	CPI	VPI	VPR
.91	.84	92	.08	01	.32	.07	.01	48
.98	.94	.91	.07	.02	.63	07	.02	.57
.99	.89	.83	.06	04	.58	06	.04	48
.95	93	92	03	03	.64	.03	.03	.59
.97	.87	.96	.07	.08	1.03	.06	.07	.99
.95	.97	.92	08	.01	94	.08	.01	.86
.97	.88	.94	.05	04	.59	.05	.04	.55
96	.82	.96	.03	10	70	.03	.08	67
.98	.95	95	01	.01	67	.01	.01	.62
.98	.98	.97	03	.01	.50	03	.01	49
.97	.96	.97	.01	.02	77	01	.02	75
.96	.93	.93	.01	.02	66	01	.02	.61
.97	.89	.97	.02	04	72	.02	.04	70
.98	.99	.95	.01	.00	45	.01	.00	43
.94	.96	.97	06	.03	.84	06	.03	.81
Median								
.97	.93	.94	.03	.02	.66	03	.02	.61

Control (median) 219.
CPI Cotton Pad Impression Method.
VPI Vel et Pad Impression Method
VPR: Velvet Pad Rinse Technique

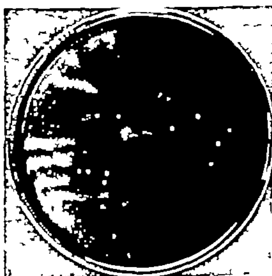


Fig 7 Experiment C. (Fig 3 plate II) —Colonies of *S epidermidis* developed 24 h after transfer of colony-forming units by imprints of new and reused velvet pads on plate II

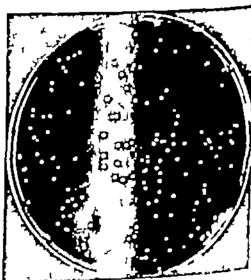


Fig 8 Experiment C (Fig. 4 plate II) —Bacterial recovery of *S epidermidis* on plate II (24 h incubation at 37 C) after the velvet pad rinse technique

TABLE 3 *Experiment D Comparison of the Median Effective Transfer of* S epidermidis *after Storage of the Velvet Pads in Saline for 24 h at 4 C and after no Storage*

Number of experiments	Estimation of transfer (Median)					
	Uptake		Release		Effective	
D	0 h	"24 h"	0 h	24 h	0 h	4 h
14	.92	.89	.64	4	.59	.38

Control (median) 188.

TABLE 4 *Experiment E. The Effective Transfer of a Mixed Culture of* S. epidermidis *and* E. coli *after Storage of the Velvet Pads Saline for 2 h at Room Temperature Compared t no Storage*

Estimation of transfer											
Uptake				Release				Effective			
0 h		"2 h		0 h		2 h		0 h		2 h	
	c		c	s	c	s	c	s	c	s	c
.98	.93	1.00	1.00	.83	43	.54	47	.81	40	.54	47
.99	.96	.99	1.00	.50	17	.34	.38	.50	16	.34	.38
1 00	1.00	.98	.87	.66	53	38	.35	66	.53	.37	.30
.99	.95	.99	1.00	49	1.28	.53	1.53	49	1.22	.52	1.53
99	.94	99	1.00	.38	40	41	.69	.38	.38	41	.69
.99	1.00	.98	91	.58	74	41	.29	.57	74	40	.26
.94	.97	.98	1.00	.53	17	.33	42	.50	16	.52	42
.99	.91	1.00	.64	.50	1.00	.50	3 14	.50	.91	.50	2.01
.99	.97	.98	.94	40	45	.33	17	40	44	.32	16
.99	1.00	.95	.92	43	62	.55	79	43	62	.52	73
.98	.96	.87	.88	.62	76	.65	43	.61	73	.57	.38
.97	.81	99	1.00	.50	.64	.53	2.00	49	.52	.52	2.00
.99	1.00	.99	.89	92	1.53	.60	1 76	.91	1.53	.60	1.57
.98	1.00	.95	1 00	42	73	.64	.93	40	73	.61	.93
.99	1.00	.97	.96	72	17	.59	18	71	17	.57	17
					Median						
.99	.97	.98	.96	.50	.62	.53	47	.50	.53	.52	47

Control (median): 128 (s) 23 (c)
s: *S. epidermidis*
c: *E. coli*

+4 C and after no storage was statistically significant ($p<0.01$) Thus, the proportional decrease which can be ascribed to storage of the pads *per se* equals 35.6 per cent.

Experiments E and F—The very few colonies to develop from the mixed culture of *S epidermidis* and *E. coli* which remained after velvet pad sampling, are seen in Fig. 9 The medians for effective transfers were 0.50 and 0.53 for staphylococci and coli, respectively and after 2 h storage in saline at room temperature, 0.52 (*S epidermidis*) and 0 47 (*E. coli*) (Table 4) The differences between storage and no storage were not statistically significant ($p>0.05$) After storage of the moistened velvet pad in a dry sterile Petri dish for 2 h at room temperature (experiment F) the median effective transfer decreased significantly from 0.53 to 0.27 (*S epidermidis*) and from 1.00 to 0 10 (*E. coli*)

TABLE 5 *Experiment F The Median Effective Transfer of a Mixed Culture of S epidermidis and E. coli after Storage of the Velvet Pads in a Petri Dish for 2 h at Room Temperature Compared to no Storage*

Number of experiments	Estimation of transfer											
	Uptake				Release				Effective			
	0 h		"2 h"		0 h		2 h		0 h		2 h	
	s	c	s	c	s	c	s	c	s	c	s	c
F												
15	.97	1.00	.97	96	.57	1 00	.28	11	.53	1.00	.27	10

Control (median): 150 (s) 26 (c)
s *S epidermidis.*
c *E. coli.*

(Table 5) The proportional decrease, solely ascribed to storage, equals 49 per cent in the case of *S epidermidis* and 90 per cent in the case of *E. coli*.

No growth was detected in 13 control pads which were taken through all experimental procedures.

Experimental Statistics

Forty randomly selected velvet pads had their 4 sides and 2 diagonals measured (sliding gauge) for control of the area, which was estimated in two ways, using the formula, $A = \sqrt{s(s-a)\ (s-b)\ (s-c)}$ and the 2 diagonals, respectively The mean area was 910.1 mm² and the standard deviation 21.6 mm² Most data have been given in a summarized form by means of median values. The counts deviated more in experiments with a mixed bacterial culture, but no statistically significant difference could be demonstrated between the remaining colony counts in ex

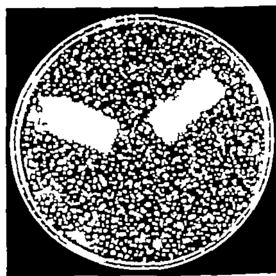


Fig 9 Experiment E.—Bacterial test surface of *S epidermidis* and *E. coli* 24 h after velvet pad sampling.

TABLE 6. *The Efficiency and Reproducibility of the Velvet Pad Rinse Technique as Determined by the Effective Transfers of* S. epidermidis *in Four Experiments*

Effective transfer in experiments			
C	D	E	F
48	44	81	55
57	63	50	61
48	45	66	40
59	54	49	52
99	69	38	56
86	91	57	56
55	66	50	50
67	71	50	69
62	46	40	73
49	48	43	50
75	76	61	58
61	49	49	55
70	45	91	81
43	110	40	47
81		71	81
Median			
61	59	50	55

periments C, D E and F From experiment C onward, when the rinse and shake procedure was introduced, this procedure was used as a reference in the subsequent experiments. Table 6 thus gives the estimated effective transfer of *S epidermidis* in 4 experiments. A non-parametric analysis of variance (*Kruskal Wallis*) demonstrated no statistically significant differences, even though the medians were lower in the experiments with a mixed culture (E and F)

DISCUSSION

Efficiency of Bacterial Transfer

Uptake.—The changes in technique from experiment A to B must be considered responsible for the increases in median uptake by new and re-used pads, both velvet and cotton, in particular the immediate sampling from the test surface after visible moisture had evaporated. Cotton pads were not investigated further because these pads would be too soft for many sampling purposes. Throughout all the experiments, including those with a mixture of *S epidermidis* and *E coli*, the uptake was close to 100 per cent. Earlier *Lederberg & Lederberg* (1952) had estimated values for the uptake of colonies. They considered that only 10 to 30 per cent of the initial cells were taken up by velvet.

Release.—The impression technique released only 1 to 4 per cent of bacteria from the velvet pad in the present experiments, in agreement with findings by *Elek & Hilson* (1954) but lower that those reported by *Lederberg & Lederberg* (1952) Striking, however was the increase in release of bacteria after rinsing of the velvet pads, resulting in a 30-fold improvement in the recovery rate from 2 to 61 per cent. Previous studies have shown that rinsing procedures yield higher bacterial recoveries than the cotton swab or agar contact methods, for example (*Angelotti et al.* 1958)

Influence of Storage on Bacterial Recovery

In an attempt to facilitate the transportation of clinical samples, the moistened velvet pad was stored in a sterile Petri dish for 2 h after sampling, but the median effective transfer decreased significantly for both *S epidermidis* and for *E. coli* in line with an earlier study on coverslips (*Pettit & Lowbury* 1968) After velvet pad storage at +4 C for 24 h, a moderate but significant decrease in median effective transfer of *S epidermidis* was seen. Storage of the velvet pad in 0.9 per cent saline for 2 h at room temperature had no significant influence on the median effective transfer of *S epidermidis* and *E. coli*. This contradicts to some extent an earlier study by *King & Hurst* (1963) where the mortality after 120 min was 99 per cent in the case of *S aureus Streptococcus pyogenes* and *E. coli*, if broth cultures were diluted in 0 85 per cent saline. Ringer's solution was also found unsuited, and only 0 1 w/v per cent peptone water seemed to be a satisfactory diluent, although limited growth could occur In the present experiments, the velvet pad may have transferred som nutrients to the saline, and this may be the reason why recovery rates were equal whether or not storage for 2 h had taken place.

Reproducibility of Bacterial Transfer

When bacteria are randomly and independently distributed their behaviour is predictable from the binomial distribution and its approximations, the *Poisson* and the normal distribution (*Meynell & Meynell* 1970) On a contaminated surface, however the distribution of the bacterial population is not *Gaussian* but considered by some to be approximately log normal (*Hansen* 1962) Greater differences tend to occur when mixed bacterial cultures are plated. For this reason, distribution-free statistical tests were used, which do not imply normality as regards the distribution from which the samples are drawn at random. An ordinary measure of scatter for the further characterization of the non-parametric distribution is not available at present, but this has to be described by observed quantiles e.g 1/6 and 5/6 The *Kruskal Wallis* variance analysis of the esti

mated effective transfers demonstrated no statistically significant differences between 4 experiments. This means that no differences were found, other than those accounted for by chance, between experiments with daily controls on different levels, the 1/6 and 5/6 quantiles for all 59 experiments being 44–45 and 76–81 The velvet pad rinse technique, as introduced, may thus be said to be a satisfactory sampling method and the fairly constant high recovery of bacteria in these experiments could be a basis for its use in the clinical routine.

I am grateful to Miss *C Zak* for her expert technical help and to *J Nyboe* statistician Office of Statistics Rigshospitalet, for advice with the statistical assessments

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REFERENCES

Angelotti R., Foter M J Busch K A & Lewis K H A comparative evaluation of methods for determining the bacterial contamination of surfaces. Food. Res. 23 175–185 1958.

Bradley J V Distribution-free statistical tests. Prentice Hall, Inc., New Jersey 1968, p. 96–114

Elek S D & Hilson C R F. Combined agar diffusion and replica plating techniques in the study of antibacterial substances. J clin. Path. 7 37–44 1954

Gorrill R. H & Penikett E. J K. New method of studying the bacterial flora of infected open wounds and burns. Lancet 2 370–372, 1957

Hansen N -H. A simplified method for the measurement of bacterial surface contamination in food plants and its use in the evaluation of pressure cleaners. J appl. Bact. 25: 46–53, 1962.

Holt R. J Pad culture studies on skin surfaces. J appl. Bact. 29 625–630 1966.

Holt R J. Aerobic bacterial counts on human skin after bathing. J Med. Microbiol. 4 319 327 1971

King W L & Hurst A A note on the survival of some bacteria in different diluents. J appl. Bact. 26 504–506 1963

Lederberg J & Lederberg, E. Replica plating and indirect selection of bacterial mutants. J Bact. 63 399–406 1952.

Meynell G G & Meynell E Theory and practice in experimental bacteriology 2. ed. Cambridge University Press, London 1970 p. 1 4

Pettit F & Lowbury E. J L. Survival of wound pathogens under different environmental conditions. J Hyg (Camb) 66 393–406, 1968

Raahave D Bacterial density in operation wounds. Acta chir scand. 140 585–593 1974.

Reibel C Gr uhaus E Mrack R. & Basset A Bactériologie de la peau saine Bull. Soc. franc Derm. Syph 74 308–311 1967

Saggers B. A & Stewart G. T. Polyvinyl-pyrrolidone-iodine A assessment of antibacterial activity J Hyg. (Camb) 62 509–518, 1964

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DETERMINATION OF MEASLES VIRUS-SPFCIFIC NUCLEOCAPSID ANTIBODIES BY MEANS OF COUNTERIMMUNOELECTROPHORESIS

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Nordal, H. J., Vandvik, B. & Norrby, E. Determination of measles virus-specific nucleocapsid antibodies by means of counterimmunoelectrophoresis. Acta path. microbiol. scand. Sect. B, 83: 425–432, 1975.

A counterimmunoelectrophoretic method for quantitative determination of antibodies against measles virus nucleocapsid is described. Unfractionated material from measles virus infected cell cultures was used as antigen, diluted to give only a nucleocapsid-specific precipitate. Purified nucleocapsid and a rabbit hyperimmune serum against this antigen was used to establish the specificity of the test. The measles virus specific antibody titres determined by the method were comparable to those found in nucleocapsid complement fixation tests. It is concluded that the technique described offers a simple and sensitive method to determine nucleocapsid-specific antibodies.

Key words: Measles virus-specific nucleocapsid antibodies; counterimmunoelectrophoresis.

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Antibodies to three separate measles virus structural antigens, haemagglutinin (HA), haemolysin (HL) and nucleocapsid (NC) have been demonstrated in man after measles virus infection (16, 17, 20, 21). Antibodies to the NC antigen can be determined by complement fixation (CF) using purified NC antigens (15, 16) or by passive double immunodiffusion using unfractionated cell-associated measles virus materials (16, 24). The latter method suffers from a relatively low sensitivity, while the former entails a somewhat laborious purification of the NC antigen. The present paper reports on the use of counterimmunoelectrophoresis* as a simple and convenient method for the determination of measles virus NC antibodies. The method allows the use of unfractionated cell-associated virus material as antigen, while retaining a sensitivity and specificity comparable to the NC CF method.

* Synonyms found in the literature: counterelectrophoresis, counter current electrophoresis, crossing over electrophoresis, immunoosmophoresis, immunoelectroosmophoresis, electroimmunodiffusion, electrosyneresis.

MATERIALS AND METHODS

Antigens. A pool of measles virus cell associated ("cell pack") antigen was prepared from Vero cell cultures infected with the Edmonston A strain of measles virus. The multiplicity of infection was approximately 1. The cultures were grown in medium 199 supplemented with 2 per cent calf serum. They were harvested by treatment with trypsin after 7–10 days, at which time the cell morphology was dominated by giant cells and cell syncytia. The cells were washed three times in medium and centrifuged at 1 000 × g for 5 min. The cell pellet was resuspended in phosphate-buffered saline (PBS) to a 10 per cent (v/v) suspension. The cell suspension was freeze thawed five times and then sonicated three times at 20 kHz, 10 sec each time. Control antigen was prepared from non-infected Vero cell cultures in the same way. (The measles virus and control cell pack antigens were kindly supplied by Dr *Miklos Degré* Kaptein W. Wilhelmsen og Frues bakteriologiske Institutt, Rikshospitalet, Oslo, Norway). Other antigens used were purified NC and small particle HA prepared as described previously (16) from Vero cell cultures infected with the LEC strain of measles virus, isolated from a patient with subacute sclerosing panencephalitis. All antigens were stored at –20° C and sonicated for 5 sec before use.

Sera. Monospecific rabbit antisera to measles virus NC and small particle HA were prepared as described previously (16). Among the human sera studied were sera from 2 patients with subacute sclerosing panencephalitis (SSPE), from 43 patients with definite (22) multiple sclerosis (MS), from 2 healthy adults, together with a pool prepared from sera of 25 adult healthy blood donors. Sera without detectable haemagglutination inhibiting (HI) antibody activity from 2 children without a clinical history of measles were used as negative control sera.

Counterimmunoelectrophoresis. Glass plates were coated with an 0.9 mm layer of 1 per cent agarose (Agarose A 37, L'Industrie Biologique Française) in a barbital buffer (LKB – Beckman Instruments AB, S-161 11 Vällingby 1, Sweden) of pH 8.6 and an ionic strength of 0.05. Double rows of opposing wells, 3 mm in diameter and 8 mm (centre/centre) apart, were punched in the gel. 5 µl volumes of antigen were applied in the cathodal wells and similar volumes of sera in the anodal wells. The antigen proved to be unstable in the diluted form and was therefore always used within a few hours after final preparation. Electrophoresis was run at 12 V/cm for 45 min, using a water-cooled apparatus (13) and the barbital buffer described above. The plates were fixed in picric acid, dried, stained with Coomassie brilliant blue for 20 min and destained in an acetic acid-methanol solution. Using sera with titres lower than 8 or 16, washing of the plates in PBS for 24 h was necessary as the staining of other gammaglobulins obscured the precipitates. Control experiments with sera of varying titres showed that the washing procedure did not change the antibody titres recorded.

Other methods. Crossed immunoelectrophoresis was performed essentially as described by *Weeke* (26) and semiquantitative determination of measles virus NC antibodies by passive double immunodiffusion as described elsewhere (24). Measles virus HI, HL inhibition (HLI) and NC CF antibodies were determined as described previously (4, 15, 16).

Statistical analysis. The various measles virus antibody titres were expressed as their $\log_2$ values. Linear regression analysis and calculation of the correlation coefficients were made using the following formulas:

regression equation

$$Y = \bar{Y} (bX - \bar{X}) \text{ where } b = \frac{\Sigma (X - \bar{X}) (Y - \bar{Y})}{\Sigma (X - \bar{X})^2}$$

correlation coefficient

$$\frac{\Sigma (X - \bar{X}) (Y - \bar{Y})}{\sqrt{\Sigma (X - \bar{X})^2 \, \Sigma (Y - \bar{Y})^2}}$$

The 95 per cent confidence intervals of the correlation coefficients were read from a table on confidence belts (7).

RESULTS

Mobility of antibody and antigen. Immunoelectrophoresis of samples containing antibody against measles virus cell pack antigen showed that the gel-precipitating (GP) antibody activity migrated cathodally (Fig. 1).

Crossed immunoelectrophoresis indicated that the cell pack antigen contained a major fraction of precipitating antigens with fast anodal mobility and minor fractions with less anodal mobilities (Fig. 2). The purified measles virus NC antigen preparation appeared to be more homogeneous and the major peak corresponded roughly in mobility to the major fraction of the cell pack antigen (Fig. 2). Careful examination of the precipitate patterns indicated a physicochemical heterogeneity of both the cell pack and the purified NC antigens. The precipitates appeared to be made up of two to three distinct lines converging and splitting along the electrophoretic spectrum (Fig. 2).

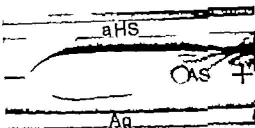


Fig 1 Immunoelectrophoresis of an SSPE serum (AS) against measles cell pack antigen (Ag) and an antiserum from a rabbit immunized with human serum (aHS) The strong precipitate arc against the latter represents IgG

Standardization of the counterimmunoelectrophoretic assay With the exception of the two negative control sera, all the human sera formed one precipitate line if tested against undiluted cell pack antigen. Some of the sera, particularly the high titred ones, formed more or less well-defined additional lines. These lines were not visible if dilutions were higher than 1:32 of antigen, or 1:16 of the sera. No precipitates were formed with uninfected Vero cell cultures as antigen.

In a series of experiments, different dilutions of antigen were tested against various dilutions of an SSPE serum (Ru) The sensitivity for detection of antibody and antigen increased with increasing dilutions of antigen and antibody respectively Dilutions of the antigen of 1:256 or higher however resulted in a marked prozone phenomenon (Fig 3) Furthermore, at these high dilutions of antigen, the precipitates were weak and difficult to discern. A 1:128 dilution of antigen was chosen for the standard titration procedure in order to obtain a sensitivity as high as possible. Since this dilution was only one step below the dilution giving prozone reaction and weak precipitation, an SSPE serum with high titres of measles antibodies was included as a reference in all experiments for calibration and quality control of the antigen. Tests of various sera showed that titres recorded under these conditions were 4 to 16 times higher than those of passive double immunodiffusion tests (Table 1) An antigen dilution of 1:64 has been used in the later routine application of the method, with approximately the same results.

As shown in Fig. 4 the precipitate at the dilution taken as end point was easily distinguishable from the negative reaction of the following serum dilution. This simplified the reading of tests.

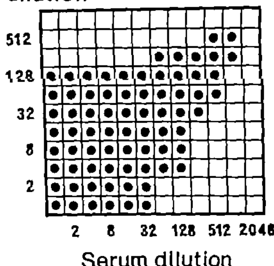


Fig 3 Precipitation (dark circles) as a function of the dilution of antigen and of serum. Cell pack antigen was examined against serum from a patient (Ru) with subacute sclerosing panencephalitis.

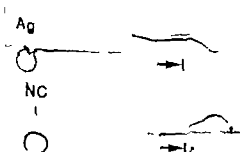


Fig 2 Crossed immunoelectrophoresis of cell pack antigen (Ag) and purified measles nucleocapsid antigen (NC) against serum from a patient (Ru) with subacute sclerosing panencephalitis. The arrows indicate the mobility of human serum albumin.

TABLE 1 *Assay of Measles Virus Nucleocapsid Antibodies in Human Sera by Passive Double Immunodiffusion and Counterimmunoelectrophoresis Tests**

Sera	Titre of nucleocapsid antibodies assayed by	
	Passive double immunodiffusion	Counterimmuno-electrophoresis
Control sera§	<1	<4
Adult human serum Va	<1	8
Adult human serum No	16	128
Pooled human serum (25 adult healthy blood donors)	4	16
MS patient Ka	1	8
MS patient Te	4	64
SSPE patient Ru	64	51
SSPE patient So	128	1024

* The standard measles cell pack antigen was employed diluted 1 8 for the former and 1 128 for the latter method.

§ Sera without detectable haemagglutination inhibiting (HI) activity from two children without a clinical history of measles.

It applies to all serum samples that the end point precipitate was formed at the same dilution step if the electrophoresis time was varied between 30 and 120 min. A lower dilution at the end point was usually recorded when the time was reduced to 15 min, even though some high titred sera showed no difference. A period of 45 min was therefore chosen as standard time for the running of electrophoresis tests.

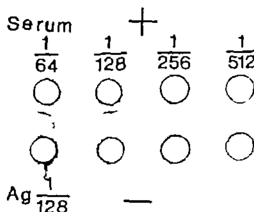


Fig. 4 Counterimmunoelectrophoresis with cell pack antigen (Ag) and a serum sample. The plate was not washed before fixing and staining

A discontinuous buffer system with lowered ionic strength in the gel has been reported to increase the sensitivity of counterimmunoelectrophoresis in some systems (5 23) An ionic strength in the gel buffer of approximately one half and one fourth of the buffer used in the electrode chambers was therefore tried, but this modification resulted in a reduction of the sensitivity of the test. In another attempt to increase the sensitivity the pH of the buffer system was lowered to 6.2 to see whether a reduction of the anodal mobility of the antigen and an increase of the cathodal mobility of the antibodies would have the desired effect. This was not the case, and carried the disadvantage of leading to the deposition of lipoproteins between the wells, obscuring the precipitates in unwashed plates.

Specificity of the precipitin reactions. A rabbit hyperimmune serum against purified measles virus NC reacted with only one sharp and well-defined precipitate line against the cell pack antigen. The relation of this precipitate to the main precipitate obtained with human sera was further studied in experiments in which the serum samples were allowed to diffuse into the gel prior to the counterimmunoelectrophoresis. Examples of

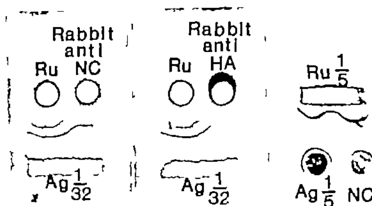


Fig. 5 a–c. Identification of the precipitate line developed by counterimmunoelectrophoresis. Rabbit antisera against measles virus nucleocapsid (Rabbit anti-NC) and haemagglutinin (Rabbit anti-HA) were allowed to prediffuse in the gel for 5 h (Fig. 5a and b) the cell pack antigen (Ag) and purified nucleocapsid antigen (NC) for 24 h (Fig. 5c) before application of antigen or serum in the opposing through and start of electrophoresis. Sera and antigen were used undiluted unless otherwise stated.

the results obtained by these prediffusion experiments are shown in Figs. 5 and 6. The line developed by the rabbit anti-NC antiserum showed a reaction of identity with the major line developed by the SSPE reference serum (Ru) (Fig. 5a). A high titred rabbit anti HA antiserum formed no precipitate against the cell pack antigen, and did not interfere with the major precipitate line of the SSPE serum (Fig. 5b). One precipitate line only was obtained by SSPE and other sera against the purified NC antigen. This line showed a reaction of complete identity with the precipitation lines formed by the same sera against the cell pack antigen (Fig. 5c).

Prediffusion experiments using a number of sera with different antibody titres, including the SSPE reference serum (Ru), showed a reaction of identity between the precipitates formed (Fig. 6). The combined results indicated that the antibodies against measles virus cell pack antigen detected in human sera under the conditions employed were antibodies to the virus NC. They will therefore in the following be referred to as measles virus NC GP antibodies.

Comparisons between measles virus NC GP antibodies and NC CF, HI and HLI antibodies in sera from patients with MS. To provide further evidence of the specificity of NC GP antibodies determined by counterimmunoelectrophoresis, and also to compare the sensitivity of the method with the NC CF technique, sera from 43 patients with MS were tested for measles virus HI, HLI, NC CF and NC GP antibody titres. Scatter dia-

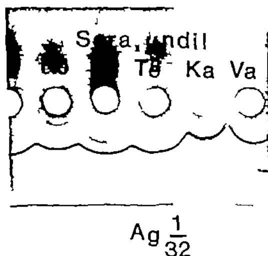


Fig. 6. Counterimmunoelectrophoresis of various sera (see Table 1) with different titres of gel precipitating antibodies against the measles virus cell pack antigen (Ag). The sera were allowed to prediffuse for 5 h before start of electrophoresis.

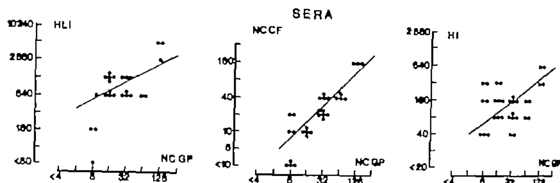


Fig 7 Scatter diagrams of the relation of nucleocapsid gel precipitation (NC GP) titres to haemagglutination inhibition (HI) haemolysis inhibition (HLI) and nucleocapsid complement fixation (NC CF) titres in sera from 43 patients with multiple sclerosis. The lines drawn represent the regression equations between the variables.

grams of the titre relationships are shown in Fig 7

The correlation coefficient between the NC GP and NC CF titres was 0.86 (95 per cent confidence interval 0.8–0.9) This was considerably higher than the correlation coefficient of 0.55 (0.3–0.9) between NC GP and HI titres, and of 0 48 (0.2–0 7) between NC GP and HLI titres. These results were in further support of the evidence that the counterimmunoelectrophoretic method and the NC CF technique assayed the same antibody populations in the sera. The correlation coefficient between the two together with the regression equation ($Y = 0.91 X + 0.35$ where $Y = \log_2$ NC CF titre and $X = \log_2$ NC GP titre) indicated that the sensitivity of the two methods was approximately the same.

DISCUSSION

Counterimmunoelectrophoresis has been used mostly for the qualitative demonstration of antigen (2, 3 5 6 8, 9 11 14 23 25) or antibody (1 12, 18) The present results show that this method may conveniently be employed as a semiquantitative assay of human antibodies to measles virus NC antigens by the use of the cell pack antigen described here This material contains considerable amounts of viral NC structures (16)

Antibodies against NC structures dominate quantitatively among antibodies to appear in connection with and to persist after regular measles infection as demonstrated by complement fixation and immunodiffusion tests (15 21) In agreement with these observations, the dominant precipitate line obtained by the cell pack antigen was shown to be formed by NC-specific antibodies. This conclusion was based on the reaction of identity with NC-specific rabbit antibodies, and on a high correlation between the titres obtained by counterimmunoelectrophoresis and those obtained by NC CF tests of the same serum samples.

Some of the sera especially the high-titred ones from the 2 SSPE patients, developed additional precipitation lines against the measles virus cell pack antigen. The additional antigenic components involved in their formation were not identified. They seemed to be present in relatively low amounts, as they were not detected by diluted antigen.

Previous immunodiffusion experiments with NC structures from myxo-and paramyxoviruses (10 15 19) have shown the occurrence of multiple lines of precipitation. The heterogeneity of the cell pack antigen and of the purified NC observed here by crossed immunoelectrophoresis is in accordance with these findings.

The possible use of counterimmunoelectrophoresis for quantitative purposes has been discussed in some studies (1 6, 9 12 14 18,

23). A prominent feature of the method, in comparison with passive double immunodiffusion, is the liability of precipitate formation, resulting in false negative reactions both in antigen and antibody excess (14, 18, 23). This is in agreement with our experience concerning the importance of using carefully calibrated concentrations of antigen, and running the sera in several dilutions.

Recent works have shown that various neurological diseases may exhibit a differentiated antibody response to different measles virus antigenic components, and it has been increasingly emphasized to employ a panel of antibody assay systems, including a CF test with purified NC antigens (15, 20, 21). The present work shows that measles NC antibodies in titres comparable with those obtained by the more complicated and time-consuming CF technique can be obtained by counterimmunoelectrophoresis against a crude cell pack antigen. In some respects, this inexpensive and simple method has advantages over the CF technique, e.g. when the test sample has a strong anticomplementary activity or when only microvolumes are available.

REFERENCES

1. *Blaller, J. R. J.* Demonstration of precipitins against a treponemal antigen by counterimmunoelectrophoresis. Lancet *I* 996–997, 1972.
2. *Bramuth, M. R., Llewellyn, P. & Jacobs, A.* A rapid method for the detection of ferritin in serum. J. clin. Path. *24* 581–582, 1971.
3. *Bussard, A.* Description d'une technique combinant simultanément l'électrophorèse et la précipitation immunologique dans un gel: l'électrosynérèse. Biochim. biophys. Acta (Amst.) *34* 258–260, 1959.
4. *Chiarini, A. & Norrby, E.* Separation and characterization of products of two measles virus variants. Arch. ges. Virusforsch. 29 205–214, 1970.
5. *Cambridge, B. S. & Shaw, Constance.* Immunoelectroosmophoresis using discontinuous buffer system to detect Australia antigen in pooled human plasma. Lancet *ii* 1184–1185, 1971.
6. *Dorff, G. J., Coonrod, J. D. & Rytel, M. W.* Detection by immunoelectrophoresis of antigen in sera of patients with pneumococcal bacteraemia. Lancet *I* 578–579, 1971.
7. *Dunn, Olive Jean.* Basic statistics: A primer for the biomedical sciences. John Wiley & Sons Inc. New York, London, Sidney, 1964, p. 175.
8. *Edwards, E. A.* Immunologic investigations of meningococcal disease. I. Group-specific *Neisseria meningitidis* antigens present in the serum of patients with fulminant meningococcemia. J. Immunol. *106* 314–317, 1971.
9. *Gock, D. J. & Howe, C.:* Rapid detection of Australia antigen by counterimmunoelectrophoresis. J. Immunol. *104* 1031–1032, 1970.
10. *Hans, L. & Hoyle, L.* The disintegration of the internal ribonucleoprotein of influenza virus A with the production of serologically distinct components. Acta virol. *10* 506–512, 1966.
11. *Hierholzer, J. C. & Barme, M.* Counterimmunoelectrophoresis with adenovirus type-specific anti-hemagglutinin sera as a rapid diagnostic method. J. Immunol. *112* 987–995, 1974.
12. *Jameson, J. E.* Rapid and sensitive precipitin test for the diagnosis of farmer's lung using immunoosmophoresis. J. clin. Path. *21* 376–382, 1968.
13. *Johansson, B. G.* Agarose gel electrophoresis. Scand. J. clin. Lab. Invest. *29* Suppl. 124, 7–19, 1972.
14. *Kohn, J.* Method for the detection and identification of alpha fetoprotein in serum. J. clin. Path. *23* 733–735, 1970.
15. *Norrby, E. & Gollmar, Ylva.* Appearance and persistence of antibodies against different virus components after regular measles infections. Infect. Immun. *6* 240–247, 1972.
16. *Norrby, E. & Hammarskjöld, B.* Structural components of measles virus. Microbios. *5* 17–29, 1972.
17. *Norrby, E. & Gollmar, Ylva.* Identification of measles virus-specific haemolysis-inhibiting antibodies separate from haemagglutinating-inhibiting antibodies. Infect. Immun. *11* 231–239, 1975.
18. *Remington, J. S., Gaines, J. D. & Gilmer, M. A.* Demonstration of Candida precipitins in human sera by counterimmunoelectrophoresis. Lancet *I* 413, 1972.
19. *Rott, R.* Untersuchungen über die Feinstructur der infektiösen Partikeln der New-castle Disease Virus und über die neben ihm auftretenden, nichtinfektiösen, virusspezifischen Einheiten. Zentralbl. Veterinärmed. *12–13* 74–116, 1965.
20. *Salmi, A., Gollmar, Ylva, Norrby, E. & Panelius, M.:* Antibodies against three different structural components of measles virus in patients with multiple sclerosis, their sibl-

ings and matched controls. Acta path. microbiol. scand. Sect. B *81* 627–634 1973

21 *Salmi A Norrby E. & Panelius M.*, Identification of different measles virus-specific antibodies in the serum and cerebrospinal fluid from patients with subacute sclerosing panencephalitis and multiple sclerosis. Infect. Immun. *6* 248–254 1972.

22. *Schumacher G A., Beebe G., Kibler R. F., Kurland L. T., Kurtzke J F., McDowell, F., Nagler Benedict Sibley W A., Tourtellotte W W & Willmon T L.*. Problems of experimental trials of therapy in multiple sclerosis Report of the panel on the evaluation of experimental trials of therapy in multiple sclerosis. Ann. N Y Acad. Sci. *122* 552–568, 1965

23 *Tripodi D Kochetsky R., Lyons Susan & Davis O* Immunochemistry of counter immunoelectrophoresis and the effect of electro-endosmotic flow on reactivity J Immunol Methods *4* 1–10 1974

24 *Vandvik, B. & Degré M.*. Measles antibodies in serum and cerebrospinal fluid in patients with multiple sclerosis and other neurological disorders with special reference to evidence for measles antibody synthesis within the central nervous system. J neur Sci. *24* 201–219 1975

25 *Wallis C & Melnick J L.*, Enhanced detection of Australia antigen in serum hepatitis patients by discontinuous counter-immunoelectrophoresis. Appl. Microbiol. *21* 867–869 1971

26 *Weeke B.* Crossed immunoelectrophoresis. Scand. J Immunol. 2 Suppl. 1 47–56, 1973.

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THE SEROLOGY OF *PSEUDOMONAS AERUGINOSA* ANALYSED BY MEANS OF QUANTITATIVE IMMUNOELECTROPHORETIC METHODS

III Reproducibility of a Polyvalent P aeruginosa *Reference Standard-Antigen*

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Høiby N The serology of *Pseudomonas aeruginosa* analysed by means of quantitative immunoelectrophoretic methods III Reproducibility of a polyvalent *P aeruginosa* reference Standard-Antigen. *Acta path microbiol. scand.* Sect. B, *83* 433–442, 1975

The reproducibility of a polyvalent *Pseudomonas aeruginosa* antigen (St Ag) composed of a mixture of antigens from 4 O groups of this bacterium has been studied. Ten batches of St-Ag were produced, and each of these and each of the 10 batches of antigens from the 4 strains of *P aeruginosa* were compared with St-Ag batch *1* by means of quantitative immunoelectrophoretic methods and a polyvalent antiserum (St-Ab) raised against St Ag. Fifty-three of the 55 antigens of St-Ag were stable and could be reproduced with reasonable precision in all 10 batches, and 3 of the 4 strains of *P aeruginosa* were stable in antigen composition in all batches. *One of the strains (0–5A) had lost 2 antigens in the last 5 batches, and the concentrations* of 7 other antigens were simultaneously changed, reflecting a smooth-rough dissociation. The disappearance of the 2 antigens in the latest 5 batches of 0–5A was also reflected in similar changes in the antigen composition of the latest 5 batches of St-Ag.

Key words *P aeruginosa* immunoelectrophoresis.

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The use of a polyvalent antigen extract in the investigation of the antigenic composition of *Pseudomonas aeruginosa* (*P aeruginosa*) and in the investigation of the humoral immunity in man against this species has revealed a very complex antigenic structure of *P aeruginosa* (9–15) At least 51 different common antigens are found in all strains investigated, and only 4 (variable) antigens are present in some but not in all strains (12)

The antigenic complexity of *P aeruginosa* the existence of variable antigens and the use of an antigen sample composed of extracts of 4 different O groups of this bacteria (St Ag) (9) raise the question whether such a polyvalent antigen extract can be reproduced with reasonable precision.

In the present work, 10 batches of a previously described polyvalent *P aeruginosa*

antigen (St Ag) (9) have been produced. These 10 batches have been compared qualitatively and quantitatively with the originally produced batch (St Ag batch 1) using various quantitative immunoelectrophoretic methods (3 5)

MATERIALS AND METHODS

Preparation of Antigens

Ten batches of St-Ag (designated St-Ag batch 3 to 12) were produced by sonication according to previously described principles (9) with minor modifications (11) The 10 batches were produced during one month and throughout this period, the 4 strains of St Ag were kept on Trübe media (9) at room temperature and subcultured serially once a week. During the first week the St Ag batches 3–5 were produced from the first subculture during the second week were produced St-Ag batches 6–7 from the second subculture during the third week St Ag batches 8–10 were produced from the third subculture and during the fourth week St Ag batches 11–12 were produced from the fourth subculture. Each batch of St-Ag was composed of equal amounts of antigens obtained by sonication of each of 4 different O groups of *P aeruginosa* (O groups 3 5A, 6 and 11) (9 11)



Fig 1 A

Fig 1 A & B The St-Ag/St-Ab reference pattern. *A* Crossed immunoelectrophoresis with intermediate gel of St Ag batch-1 against St-Ab in the reference gel. No antigens or antibodies were included in the intermediate gel. *B* Drawing of Fig. 1 A with enumeration of the precipitates. An arbitrary scale of migration velocity relative to human albumin (1.0) is indicated below

Fig 1 B

These 4 strains were originally mucoid, but only 8-11 has remained mucoid after storage for 3 years stabbed in meat extract agar at room temperature. The first subculture of the strains used in the present study were taken directly from these agar stabs. These strains have also been lyophilized but none of the lyophilized cultures have remained mucoid. Aliquots of antigens of each of the 4 strains from each of the 10 batches and aliquots of the 10 St Ag batches composed by mixing antigens of these strains were stored at −30° C the immunoelectrophoreses were run just after thawing of the antigen samples upon which the samples were discarded. Colloid concentrations were determined by refractometry as reported previously (11) the 10 St-Ag batches were in the range 12.3–16.0 g colloid per litre (mean 14 4 g per litre)

The St-Ag/St Ab Reference System

The St-Ag batch 1 and the corresponding polyvalent pooled rabbit antiserum (St Ab) have been described previously (8) The St-Ab used in the present work represent the pooled bleedings numbers 7 to 17 from 9 of the original 10 rabbits which have been immunized and bled for more than 3 years according to published methods (7 9) The purification and concentration of the antibodies has been described previously (7 9) When used against St-Ag batch 1 in crossed immunoelectrophoresis, the present St-Ab pool 7–17 shows a precipitate pattern similar to that originally described, without qualitative differences, the quantitative differences only being very small as judged by various quantitative immunoelectrophoretic methods (4) The antigens of *P aeruginosa* in St-Ag batch 1 have also been very stable during more than 3 years storage at −30 C. The St Ag/St-Ab reference system consists of 55 precipitates visible by means of crossed immunoelectrophoresis using the present antigen-antibody ratio. The precipitates have been enumerated, beginning with number 1 in the anodic end of the 1st dimension electrophoresis (Fig 1 A and B)

Immunoelectrophoretic Methods

Each of the 10 St-Ag batches and each of the corresponding batches of the 4 strains of *P aeruginosa* were run 1) in *crossed immunoelectrophoresis* against St-Ab, and 2) in *crossed-line immunoelectrophoresis* the antigen batch in question being included in an intermediate gel between 1st and 2nd dimension electrophoresis of St-Ag batch 1 against St-Ab (*absorption of antibodies in situ*) as described previously (11–13) Controls with saline or St Ag batch 1 in the intermediate gel were run as previously described (11–13) The percentage of antibodies absorbed in the crossed-line immunoelectrophoreses (absorption of antibodies in situ) were estimated by comparison with a set of 4 standard plates containing 100 per cent-75 per cent 50 per cent and 25 per cent of the original concentration of St-Ab in the second dimension gels as previously reported (11 12) The electrophoreses were run in microtechnique on 5 × 5 cm glass plates, using equipment, agarose and technical details as previously described (9–11) The first dimension electrophoreses were run with 2 µl of the antigens. The intermediate gels of the second dimension electrophoreses (crossed-line immunoelectrophoresis) contained 40 µl antigen per cm² or 0 154 M NaCl as control, and the reference gels of the second dimension electrophoreses contained 20 µl St-Ab per cm²

Quantitation of the Antigens

In the crossed immunoelectrophoresis, the area enclosed by a precipitate is proportional to the antigen-antibody ratio of the system (15) As the antibody amount and the volumes of the antigens were kept constant in the crossed immunoelectrophoreses, the sizes of the areas were proportional to the concentration of the individual antigens in the antigen samples. After magnification at ×9 as previously described (11) the areas were measured using a Hope Planimeter No. 9101 The mean of 2 measurements on each area was used. The reproducibility of the planimeter measurements, expressed as the relative standard deviation (16, 17) determined by double determinations on 40 different areas covering the actual range of areas, is 0.6 per cent. The reproducibility of the present modifications of the crossed immunoelectrophoresis has been determined by double-determinations of samples (16, 17) Ten pairs of crossed immunoelectrophoreses with intermediate gels were run on 10 different days and the areas enclosed by 6 different precipitates were measured. The average relative standard deviation (16, 17) (the analytical variation on different immunoplates on the same day) was 6 per cent (range 4–9 per cent) The average 95 per cent confidence limits (14) on a measurement of an area is then ± 13.4 per cent (range ± 9–21 per cent) Immunoplates which are compared quantitatively in the present work were always run simultaneously

Statistical Methods

The F-test, Student t-test and one factor analysis of variance were done using the programs of a Compucorp 342 Statistician (2, 6, 14) Level of significance 5 per cent (double-tailed tests)

RESULTS

Table 1 shows the results of comparison of the 10 batches of St Ag and the corresponding 4 strains of St Ag with the reference sy

TABLE 1 *Occurrence of 55 Antigens in 10 Batches of a Polyvalent Pseudomonas Antigen (St Ag Batch 3 to 12) and in the Corresponding 10 Batches of the 4 Strains (O Groups 3 5A 6 and 11) of Pseudomonas aeruginosa Used in St-Ag and the Corresponding Polyvalent Standard Antibody (St-Ab)*

	Antigen number (No.) and percentage of cross-reactivity in relation to the reference St-Ag/St-Ab pattern				
	No. 9 per cent	No. 18 per cent	No. 37 per cent	No. 44 per cent	Remaining 51 antigens per cent
St Ag Batches 3–7*	100	100	100	100	100
St Ag Batches 8–12	100	0	50–75	100	100
0–3 Batches 3–12	0	0	50–75	100	100
0–5A Batches 3–7	100	100	100	100	100
0–5A Batches 8–12	100	0	0	100	100
0–6 Batches 3–12	0	0	0	100	100
0–11 Batches 3–12	100	0	0	0	100

* Batches 3–7 etc. denotes that each of the 5 batches showed similar antigenic composition.

A



Fig 2 A Crossed immunoelectrophoresis of St-Ag batch 4 against St-Ab. Two arrowheads indicate 2 precipitates which are absent in Fig. 3 A (precipitate no. 18 and part of precipitate no. 37) Technical 1 dimension electrophoresis anode at the right. 2. dimension electrophoresis anode at the top. Staining: Coomassie brilliant blue)



Fig B. Crossed-line immunoelectrophoresis of St-Ag batch 1 in the w ll nd St-Ag batch 4 in the intermediate gel again t St Ab in the reference gel (absorption of antibod es *in situ*) All the precipitates of the reference pattern have been removed or elevated and corresponding straight precipitate lines showing reaction of identity with the elevated reference precipitates are seen in the gel. (The control reference pattern of St-Ag batch 1 against St Ab is not shown here but can be seen in Fig 1) Technique as in Fig. 2 A

stem. The results obtained in 2 of the batches of St Ag are shown in Figs. 2 A & 3 A. As examplified in these figures, the 10 batches of St Ag were very much alike, and the same applies to the batches of 3 of the strains of St Ag (0–3 0–6 and 0–11) However some differences were found The first 5 batches of St-Ag were qualitatively completely identical with St Ag batch 1 as judged by experimental absorption of antibodies *in situ* (Figs. 2 A & B) the last 5 batches were different, *however as they could not absorb antibodies*

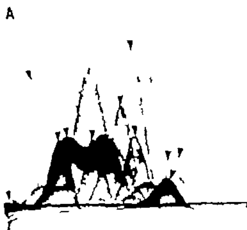


Fig. 3 A. Crossed immunoelectrophoresis of St-Ag batch 11 against St Ab. Twelve arrowheads indicate the precipitates given in Table 2. Precipitates no. 18 and part of no. 37 are absent (compare with Fig. 2 A).

against antigen number 18 and only 50–75 per cent of the antibodies against antigen number 37 (Fig. 3 A & B). Accordingly precipitates corresponding to antigen number 18 were not found in the crossed immunoelectrophoreses of St-Ag batches 8–12 against St-Ab whereas only minor changes could be seen as regards precipitates corresponding to antigen number 37 in St-Ag batches 8–12 (Figs. 2 A & 3 A). These changes in St Ag batches 8–12 were found to be due to similar changes in batches 8–12 of antigens prepared from strain 0–5A (Table 1, Fig. 4 A & B, Fig. 5 A & B) whereas the antigen composition of the 3 other strains incorporated in the St-Ag remained stable throughout the period. These results show that 2 out of the 55 antigens of St-Ag were qualitatively unstable whereas the remaining 53 antigens were stable.

The reproducibility of the antigen production procedure was studied quantitatively by quantitation of a selection of 12 representative antigens (immunoprecipitates) in all of the 10 batches of St-Ag (one immunoplate of each batch) as indicated in Fig. 3 A. By means of the F test the data were compared with those obtained by 10 repeated determinations (10 immunoplates) on one batch (Table 2). Significant differences between the variances were only found in 3 (25 per cent, 95 per cent confidence limits 5–57 per cent) of the antigens. If the colloid concentrations of the 10 batches were compared in a similar way with 10 repeated determinations on the same batch, the difference between the variances was found to be significant ($p<0.01$).

The quantitative proportions between the 12 antigens in each of the 10 batches of St Ag were calculated using the area of precipitate number 10 in each plate as unit (built in reference). The results from each of the batches were then compared by means of analysis of variance which showed no significant difference between the batches. This is in accordance with the visual impression.

The loss of 2 antigens in the strain 0–5A batches 8–12 was accompanied by quantitative changes in some other antigens (decreased

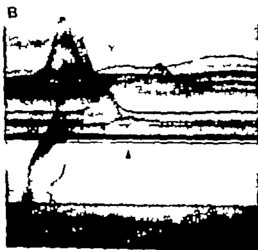


Fig. 3 B. Crossed-line immunoelectrophoresis of St Ag batch 1 in the well and St-Ag batch 11 in the intermediate gel against St-Ab in the reference gel (absorption of antibodies in situ). All the precipitates except no. 18 and 25–50 per cent of no. 37 (arrowheads) have been removed from the reference pattern (compare with Fig. 1 and Fig. 2 B). Technique as in Fig. 2 A.

TABLE 2 *Area (cm² Magnification × 9) of 12 Precipitates in the St Ag/St-Ab Pattern. Comparison of the Results from 10 Crossed Immunoelectrophoreses of 10 Different Batches of St Ag (Batch 3–12) with the Results from 10 Repeated Crossed Immunoelectrophoreses of One Batch of St Ag (Batch 12 × 10)*

		Precipitate number in the St-Ag/St Ab reference system											
		3	4	10	14	15A	15B	24	35	37	44	48	50
St Ag batches 3–12	area												
	mean	46.4	48.9	27.7	36.5	80.8	46.9	67.7	41.2	91.0	44.3	118.7	8.9
	SD	9.5	8.4	4.8	8.7	27.1	9.9	10.9	7.2	20.6	13.5	18.8	3.6
St Ag batch 12 × 10	area												
	mean	37.2	30.8	22.1	26.3	79.2	45.5	56.6	34.0	75.9	29.1	131.8	11.6
	SD	7.7	5.5	4.6	5.8	12.8	8.4	7.5	6.0	11.9	3.2	5.2	2.0
F test		n.s.	n.s.	n.s.	n.s.	+	n.s.	n.s.	n.s.	n.s.	+ +	+ +	n.s.

\+ signifies p-values below 5 per cent and + + below 1 per cent.
SD standard deviation.

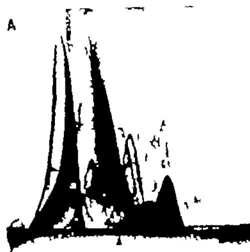


Fig. 4 A. Crossed immunoelectrophoresis of antigens from *P. aeruginosa* 0-5A batch 4 against St Ab. Two arrowheads indicate 2 precipitates which are absent in Fig. 5 A (precipitates no. 18 and no. 57)

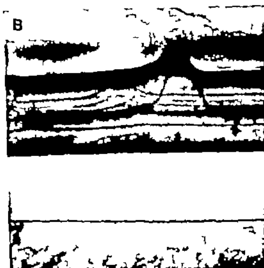


Fig. 4 B. Crossed-line immunoelectrophoresis of St Ag batch 1 in the well and 0-5A batch 4 in the intermediate gel against St-Ab in the reference gel (absorption of antibodies *in situ*). All the precipitates of the reference gel have been removed from the reference pattern. Technique as in Fig. A.

concentrations antigen numbers 9 38, 44 48 and 49 increased concentrations antigen numbers 15A and 35) whereas the concentrations of the remaining 46 antigens were not changed (Figs. 4 & 5). With a view to illustrating the significance of 2 of the observed quantitative differences, Table 3 shows the results applying to 5 of the antigens of the 10 batches of 0-5A. Similar comparisons between St Ag batches 3–7 and batches 8–12 showed no significant differences. Moreover it was noticed that, concurrent with the loss of 2 antigens and the quantitative changes concerning other antigens, the

TABLE 3. *Area (cm. Magnification × 9) of 5 Precipitates in 10 Batches of the Strain 0-5A run against St-Ab in 10 Crossed Immunoelectrophoreses. Comparison of the Results of the First 5 Batches with the Results of the 5 last Batches*

		Precipitate number in the St-Ag/St-Ab reference system				
		10	*15B*	*24*	*35*	*44*
0-5A batches 3–7	area mean	24 8	40.4	58.5	31 7	112.4
	SD	3.7	3 6	5.5	2.3	15.8
0 5A batches 8 12	area mean	28.0	43.3	62 1	40 7	39.9
	SD	1 7	7.0	2.9	1.6	9 7
F-test		n.s.	n.s.	n.s.	n.s.	n.s.
t-test		n.s.	n.s.	n.s.	+ + +	+ + +

+ + + signifies p-values below 1 per mille.
SD: standard deviation.

A



Fig 5 A Crossed immunoelectrophoresis of antigens from *P aeruginosa* 0-5A batch 9 against St Ab. Five arrowheads indicate the precipitates given in Table 3 Precipitates no. 18 and no 37 are absent (compare with Fig. 4 A)

growth of the strain 0–5A was less abundant, the yield (wet weight) decreasing by 1/3 and the colonies being of rough appearance Otherwise, the colony form of the strains remained stable throughout the study

DISCUSSION

The various quantitative immunoelectrophoretic methods were introduced into microbiology by *Axelsen* (1971) who showed that a high degree of precision and reproducibility could be obtained parallel with a hitherto unknown degree of resolving power (2) In the present work, these methods have been applied to a polyvalent antigen extract of a mixture of 4 strains of *P aeruginosa* in order to see whether such an antigen extract is also reproducible In serological studies the reproducibility of such a reference antigen sample is of crucial importance in work with such complicated reference antigen-antibody systems as the present St Ag/St Ab system.

The results show that although some differences were observed, the great majority of the antigens of St-Ag were stable and could be reproduced with reasonably quantitative precision. These findings are thus comparable with the results obtained by *Axelsen* (1973) The observed differences between St Ag batches 3–7 and batches 8–12 coud be referred to changes in antigen composition of a single strain 0–5A, incorporated in the St Ag and these changes seem to reflect a dissociation of the strain which is often seen in *P aeruginosa* (8, 18) The dissociation led to the loss of 2 antigens, one of which (no. 37) is related to the O-antigens of *P aeruginosa*, and to quantitative changes in the concentrations of a number of other antigens of 0-5A. Quantitative changes of the last-mentioned antigens in the batches of St-Ag were much smaller and not statistically significant, because of the presence of these antigens in the 3 other strains incorporated in the St-Ag After the present investigations had been carried out, 4 other batches of St-Ag (batches 13–16) were produced using the original stab cultures each time in order to avoid subculturing these batches contain all 55 antigens.

Two differences were observed when the

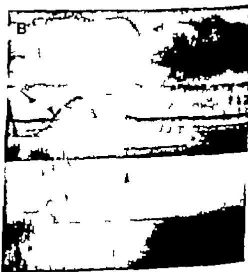


Fig 5 B. Crossed line immunoelectrophoresis of St Ag batch 1 in the well and 0-5A batch 9 in the intermediate gel against St Ab in the reference gel (absorption of antibodies in situ) All the precipitates except no 18 and no. 37 (arrowheads) have been removed from the reference pattern (compare with Fig 1 and Fig 4 B) Technique as in Fig 2 A

results of the present work were compared with previously published work (1? 13) 0-5A batches 3-7 could absorb 100 per cent of the antibodies corresponding to precipitate number 37 in contrast to 0-5A batch 1 which could only absorb 50-75 per cent of these antibodies (12) Moreover 0-11 batches 3-12 lacked antigen number 18 which was present in 0-11 batch 1 (12) As the different pools of St Ab used in previously published works (9 11-13) (pool 1 and pool 2-6) have not been found to differ from those used in the present work (pool 7-17) these differences in experimental absorption of antibodies *in situ* must reflect differences between the batches of antigens (3-5)

In the present study quantitative results are only examined as regards 12 of the 55 antigens of the St-Ag/St Ab system. These 12 antigens represent various electrophoretic mobilities and various sizes of areas enclosed by the precipitates (Fig 3 A) As judged by careful inspection of the precipitates of the remaining 43 antigens and by superposing of the plates, these 12 antigens form a representative sample of the 55 antigens of St-Ag and accordingly the conclusions of the present study can probably be extended to apply to the remaining antigens of the St Ag.

According to the results shown in the tables and the figures, the differences discussed above represented only minor problems. The St-Ag/St Ab precipitate pattern was reproducible regardless of antigen batch used and 3 of the 4 strains comprising St-Ag were stable in antigen composition throughout the study Moreover the results show that, if selection of dissociants of P *aeruginosa* (8) is avoided, the production of polyvalent antigen extracts of *P aeruginosa* can be done with high degree of reproducibil ty

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REFERENCES

1 *Axelsen N H* Antigen-antibody crossed electrophoresis (Laurell) pplied to the study of the antigenic structure of *Candida albicans* Infect. and Immun. *4* 525-527 1971

2. *Axelsen N H* Quantitative immunoelectrophoretic methods as tools for a polyvalent approach to standardization in the immunochemistry of *Candida lbicans*. Infect and Immun. *7* 949-960 1973

3 *Axelsen N H., Bock E. & Kroll J* Comparison of antigens the reaction of identity *In* Axelsen, N H., Kroll, J & Weeke, B. (Eds.): A Manual of Quantitative Immunoelectrophoresis. Uni ersitetsforlaget, Oslo 1973 p. 91-94.

4 *Axelsen N H., Bock E. & Kroll J.*, Comparison of antisera. *In* Axelsen, N H., Kroll, J & Weeke, B. (Eds.) A Manual of Quantitative Immunoelectrophoresis. Universitetsforlaget, Oslo 1973 p. 101-103

5 *Bock E. & Axelsen N H* Comparison of antigens the reaction of partial identity *In* Axelsen, N H., Kroll, J & Weeke, B. (Eds.) A Manual of Quantitati Immunoelectrophoresis. Universitetsforlaget, Oslo 1973 p. 95-99

6. *Diem K.* (Ed) *Documenta Geigy Scientific Tables.* J R. Geigy S A., Basle 1962, pp. 40-41 & 32-35

7 *Harboe N & Ingild A.* Immunization, isolation of immunoglobulins, estimation of antibody titre. *In* Axelsen, N H., Kroll, J & Weeke B. (Eds.) A Manual of Quantitative Immunoelectrophoresis. Universitetsforlaget, Oslo 1973 p. 161-164

8. *Homma J Y.*, Serological typing of *Pseudomonas aeruginosa* and several points to be considered. Jap. J Exp. Med. *44* 1 12, 1974

9 *Høiby N & Axelsen, N H.*, Identification and quantitation of precipitins against *Pseudomonas aeruginosa* in patients with cystic fibrosis by means of crossed immunoelectrophoresis with intermediate gel. Acta path. microbiol scand. Sect. B, *81* 298-308, 1973

10. *Høiby N* *Pseudomonas aeruginosa* infection in cystic fibrosis. Relationship between mucoid strains of *Pseudomonas aeruginosa* and the humoral immune response Acta path. microbiol. scand. Sect. B, *82* 551-558, 1974

11 *Høiby N* Cross-reactions between *Pseudomonas aeruginosa* and thirty-six other bacterial species. Scand J Immunol. *4* Suppl. 2, 187-196 1975

12. *Høiby N.*, The serology of *Pseudomonas aeruginosa* analysed by means of quantitative immunoelectrophoretic methods. I Comparison of thirteen O groups of *Ps. aeruginosa* with a povalent *Ps. aeruginosa* antigen-antibody

body reference system. Acta path. microbiol scand. Sect. B, *83* 321–327 1975

13 *Høiby V* The serology of *Pseudomonas aeruginosa* analysed by means of quantitative immunoelectrophoretic methods. II. Comparison of the antibody response in man against thirteen O groups of *Ps aeruginosa*. Acta path. microbiol. scand. Sect. B, *83* 328–334 1975.

14 *Therkelsen A J Medicinsk Statistik*. Akademisk Boghandel, Århus 1968, pp. 69–71 77 79 & 102–110

15 *Weeke B*., General remarks on principles, equipment, reagents and procedures. *In* Axelsen, N H., Krøll, J & Weeke, B. (Eds.) A Manual of Quantitative Immunoelectrophoresis. Universitetsforlaget, Oslo 1973 p. 15 35.

16 *Weeke B*., Rocket immunoelectrophoresis. *In* Axelsen, N H., Krøll, J & Weeke, B. (Eds.): A Manual of Quantitative Immunoelectrophoresis. Universitetsforlaget, Oslo 1973 p. 37–46.

17 *Weeke B.*: Crossed immunoelectrophoresis. *In*: Axelsen, N H., Krøll, J & Weeke, B. (Eds.) A Manual of Quantitative Immunoelectrophoresis. Universitetsforlaget, Oslo 1973, p. 47–56

18. *Zierdt C H & Schmidt J* Dissociation in *Pseudomonas aeruginosa*. J Bacteriol. *87* 1003–1010 1964

Acta path. microbiol. scand. Sect. B, 83 443–450, 1975

RAISING ANTIBODIES TO HUMAN LEUKOCYTE INTERFERON

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Human leukocyte interferon proved to be a good immunogen in sheep, rabbits and guinea pigs. Sera from immunized animals neutralized the antiviral action of leukocyte interferon at high dilution. The highest anti-interferon titres obtained were 1 1,200 000 for sheep 1:150,000 for rabbits, 1:30,000 for guinea pigs Partial purification of human leukocyte interferon, by ethanol precipitation, improved its qualities as an immunogen. While it appeared more efficient to give initial injections without adjuvant, the inclusion in booster injections, of Freund's complete adjuvant produced a markedly superior response and, in one sheep maintained high levels of circulating antibody for several months.

Key words Human leukocyte interferon anti interferon antibodies antibody production.

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Human interferons are immunogenic in animals of other species (*Levy-Koenig et al.* 1970 a *Levy-Koenig et al.* 1970 b *Dec Goran et al* 1971 *Mogensen & Cantell* 1974 *Anfinsen et al.* 1974 *Berg et al.* 1975) Chromatographic separation of interferons from their impurities, by virtue of their affinity for antibody can lead to a considerable purification of crude interferon (*Sipe et al.* 1973 *Ogburn et al.* 1973) Recently similar attempts have been made with human interferon (*Anfinsen et al.* 1974 *Berg et al.* 1975)

Human leukocyte interferon differs in some respects from interferons prepared in cultures of human diploid cells. It is more stable to heat and to variety of denaturants (*Mogensen & Cantell* 1973 *Edy et al.* 1974 *Cantell et al.* 1974) It does not require the addition of stabilizing protein after partial purification (*Cantell et al.* 1974 *Anfinsen et al.* 1974) It does not appear to be neutralized by an antiserum prepared against diploid-cell interferon, whereas an antiserum prepared against leukocyte interferon neutralizes interferon from diploid cells, though usually to a lesser degree (*Levy-Koenig et al.* 1970 a *Anfinsen et al.* 1974 *Berg et al.* 1975) It has recently been shown that human interferon crosses on bovine cells and that leukocyte interferon produces a greater antiviral effect in such cells, than does a similar dose of diploid-cell interferon (*Gresser et al.* 1974)

This paper is concerned with the preparation of potent antisera, suitable for affinity chromatography and with some of the immunogenic properties of the human leukocyte interferon used to raise the antibodies.

METHODS

Interferon. This was prepared from peripheral blood leukocytes by a modification (*Cantell et al.* 1974) of the method of *Strander & Cantell* (1966). Washed cells were suspended in Eagle's minimal essential medium, supplemented with human serum, from which most of the globulins had been precipitated with ammonium sulphate (*Cantell & Pyhälä* 1973). Cells were induced with live Sendai virus, in chick allantoic fluid. The cells were removed by centrifugation after overnight incubation at 37.5 C. The supernatant is crude interferon.

Assay of interferon. Interferon was assayed by the inhibition of vesicular stomatitis virus plaques on human amnion (U) cells (*Strander & Cantell* 1966). One unit of interferon reduces virus plaques by 50 per cent. The potency of an interferon preparation is expressed relative to the human reference interferon 69/19 (International Symposium on Standardization of Interferon and Interferon Inducers, London, 1969).

Neutralization assay. A fixed dilution of antiserum (at least 1/100) was titrated against a range of interferon dilutions (always from the same pool of crude human leucocyte interferon, dialyzed at pH 2 to destroy Sendai virus). The percentage plaque reductions were calculated and plotted versus log interferon concentration (see Fig. 1). The neutralizing titre of an antiserum is taken as the antilog of the interferon dose that gives 50 per cent plaque reduction in the presence of that antiserum, multiplied by the dilution factor (at least 100) involved (*Mogensen & Cantell* 1974). *The potencies of all antisera in this study are* expressed relative to a laboratory standard (rabbit antiserum 2/13) which was arbitrarily allotted a neutralizing titre of 1:30,000 (a value rounded off from the mean of over 30 titrations). We have exchanged antisera with Dr. A. Pecker Philadelphia who uses a different assay (*Berg et al.* 1975) and found agreement within 2-fold.

Concentration and purification of interferon. Crude interferon, precipitated by 0.5 M potassium thiocyanate at acid pH, can be resuspended to give 50-fold concentration (*Cantell & Pyhälä* 1973). Such material (designated C-IF) titres between *0.6–2.0 million units/ml and contains ca. 50 mg/ml* of protein. Little purification is achieved. The infectivity of Sendai virus is destroyed by this treatment. Partial purification of human leukocyte interferon is routinely achieved by the selective precipitation of contaminating proteins from 94 per cent ethanolic solution, at +4 C (*Cantell et al.* 1974). The final material (designated P-IF) contains ca. 10 million units/ml and ca. 30 mg/ml of protein. The conditions of purification are harsh enough to ensure that much of the protein will suffer denaturation (*Cohn et al.* 1946). Stabilizing additives are not required to maintain the biological activity of P-IF (*Cantell et al.* 1974).

Immunizations. Sheep, rabbits and guinea pigs were randomly selected for immunization with either C-IF or P-IF. Some rabbits were also immunized with "mock" interferon preparations that had been prepared similarly to interferon, except that the addition of Sendai virus was deferred until after the removal of the leukocytes by centrifugation. All injections were given subcutaneously.

Guinea pigs (ca. 600 g) were divided into eight groups of 4 or 8. Each group was submitted to one of the following schedules: weekly injections of 0.06 million units of interferon (P-IF or C-IF) or of 0.6 million units (P-IF or C-IF) without adjuvant; 4-weekly injections of 0.24 million units of interferon (P-IF or C-IF) or of 2.4 million units (P-IF or C-IF) admixed with an equal volume of Freund's complete adjuvant. Immunizations were continued for 8 to 16 weeks. All guinea pigs were regularly bled (1 ml, from the cephalic vein) and the separated serum was heated at 56 C for 30 min, prior to its titration against interferon.

Rabbits (ca. 3.5 kg) were either given weekly injections of 3 million units of C-IF or P-IF without adjuvant, or 4-weekly injections of 12 million units of P-IF admixed with an equal volume of Freund's complete adjuvant. Samples (ca. 5 ml, sometimes 30 ml) from the marginal ear vein) were treated as for guinea pigs.

Two sheep (Gunnar 35 kg & Ilvar 28 kg, both 6 months old) were initially immunized with weekly injections of crude interferon without adjuvant (16 million units over 13 weeks). This response was not very encouraging, so, after an interval of 2 weeks, they were both given weekly injections of P-IF (24 million units over 4 weeks) again without adjuvant. Subsequently boosters of 50 million units per injection (with or without adjuvant) were given. These and the subsequent responses of the sheep are described in Fig. 3 and the accompanying legend. All bleedings (ca. 10 ml, sometimes 100 ml, from the jugular vein) were treated as for guinea pigs.

RESULTS

Neutralizing Antibody

Of 40 guinea pigs, 16 rabbits and 2 sheep, only one guinea pig failed to respond to human leukocyte interferon. Neutralizing activity was often present within 4–6 weeks of starting the immunizations. The neutralizing capacity of an antiserum was approximately the same when titred against human leukocyte interferons of different purity (unpub-

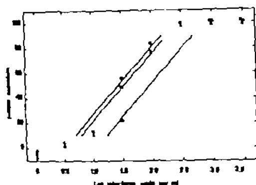
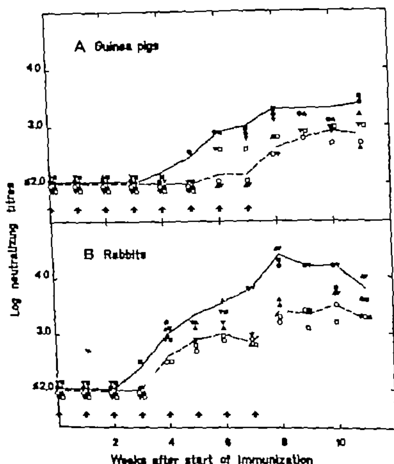


Fig. 1. The response upon log dose of interferon in the presence of 3 different antisera. ●——● Rabbit standard anti-interferon serum (2/13) ▲——▲ Sheep (Ilvar week 25) anti-interferon serum. ■——■ Sheep (Ilvar week 46) anti-interferon serum. The rabbit antiserum (1:50 000) is used as an internal standard in every assay and all titres are expressed relative to it. Thus the 50 per cent plaque depressing dose in the presence of the late-response, sheep-serum sample (■——■) is ca. 3 × (i.e. 0.5 log units) greater than that in the presence of the standard: accordingly the sheep serum sample is alloted a neutralizing titre of 1:90,000



2 The response of guinea pigs and rabbits to concentrated (C-IF) and partially purified (P-IF) erferon. A. The guinea pigs received 8 weekly injections of 0.6 million units of C-IF (open symbols) P-IF (closed symbols). B. The rabbits received 8 weekly injections of 3 million units of C-IF (open mbols) or P-IF (closed symbols).

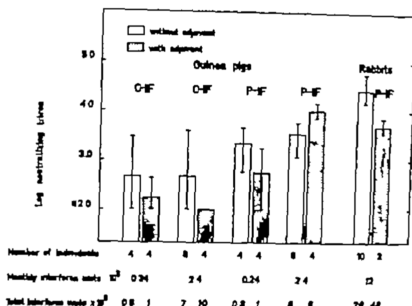


Fig 3 The effect of Freund's complete adjuvant on the antibody response of guinea pigs and rabbits to concentrated (C-IF) and partially purified (P IF) interferon. The details of the immunization schedules are given in the Methods. The log means of the highest neutralizing titres attained and the ranges of the titres in individual sera are given for each group. The total interferon units given represent the minimum doses needed to achieve the peak antibody titres in each group.

lished observations) All anti-interferon activity was precipitated from serum with the globulin fraction. Several rabbits were immunized with "mock" interferon, concentrated as for C-IF or purified as for P IF both with and without adjuvant. Some were also immunized with leukocyte extracts. None of these animals ever developed anti-interferon activity in their sera.

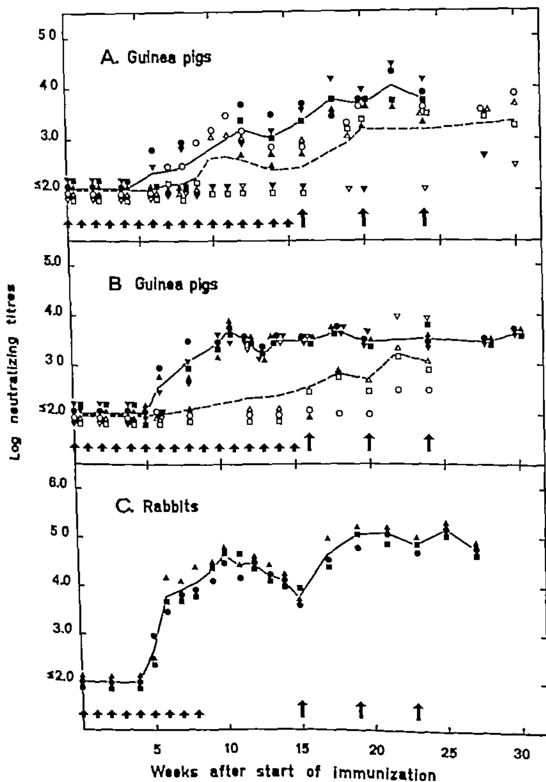
Although the mechanism by which antibody neutralizes interferon is not known, the dose response of the assay is that of interferon (*Mogensen* & *Cantell* 1974) Fig. 1 illustrates the response upon log dose of interferon, in the presence of three different antisera. The slopes are very similar which suggests that this is a valid way of estimating the relative potencies of different antisera. Four months of active antibody synthesis (Fig 5) separate the two sheep samples and any possible differences in affinity or specificity of the antibody do not appear to influence the response of the assay We have also noted that, although the earliest neutralizing antisera, may be partially sensitive to 2 mercaptoethanol, the slopes of the dose-response curves do not differ from those of later immune antisera.

Influence of Interferon Purity

The effect of the purity of the interferon preparation on the antibody response was studied by immunizing groups of 4 guinea pigs and rabbits with C-IF or P IF The interferon dose per body weight was similar in all groups. The kinetics of the appearance

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Fig 4 The enhancement of the anti-interferon response by the inclusion of Freund's complete adjuvant in booster injections. A. The guinea pig received 16 weekly injections (short arrows) of 0.06 million units of C-IF (open symbols) or P-IF (closed symbols) followed by 4-weekly booster injections (long arrows) of 2.4 million units of P-IF with adjuvant. B. The immunization schedule was as for A, except the initial weekly interferon dose was 0.6 million units. C. The rabbits received 9 weekly injections (short arrows) of 3 million units of P IF followed by 4-weekly booster injections (long arrows) of 12 million units of P-IF with adjuvant.



A. Guinea pigs
B Guinea pigs
C. Rabbits
Log neutralizing titres
5.0
4.0
3.0
≤2.0
6.0
5
10
15
20
25
30
Weeks after start of immunization

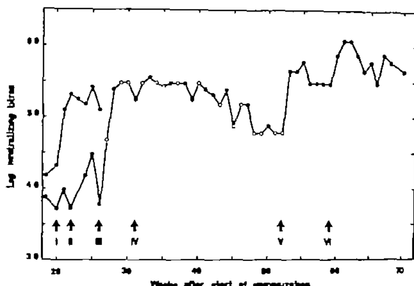


Fig 5 The effect of Freund's complete adjuvant on the development and maintenance of high levels of neutralizing antibody in sheep, immunized with human leukocyte interferon. ●—● serum samples from one sheep (Gunnar) given the following injections I & II 30 million units each of P-IF with adjuvant. ○—○, serum samples from Ilvar given the following injections I & II 30 million units each of P IF alone III & IV 30 million units each of P IF with adjuvant V 3 million units of P IF with adjuvant VI, 10 million units of P IF with adjuvant. Arrows indicate the times of injection. The initial schedules for Gunnar and Ilvar up to 19 weeks were identical (16 million units of crude interferon, over 13 weeks, then, after 2 weeks interval, 24 million units of P IF over 4 weeks)

of antibody is shown in Fig. 2. The P IF response was clearly faster and better than the C-IF response in both species. Higher antibody titres were attained in rabbits than in guinea pigs.

The Use of Freund's Complete Adjuvant

Fig 3 shows the effect of incorporating adjuvant in the immunization schedules described in Methods. Freund's complete adjuvant clearly had an adverse effect on anti-interferon production in most groups, when it was present from the start of immunization. The only exception was the group of guinea pigs which received the high dose of P IF A 10-fold increase in the dose of P IF had resulted in an improved antibody response in guinea pigs, whereas a corresponding increase in the amount of C-IF was without effect.

The incorporation of adjuvant in the booster injections was studied in guinea pigs and rabbits which had been immunized with C-IF or P IF alone. Fig 4 shows that the antibody response was enhanced in most groups after boosting with adjuvant. That the improved response is actually due to the inclusion of adjuvant seems likely from the results shown in Fig 5 The two sheep responded similarly to the same course of immunization. "Gunnar" was then given 2 injections, of 30 million units each, of P IF with adjuvant, and "Ivar" 2 injections of 30 million units each, of P IF without adjuvant. "Gunnar responded with a rapid increase in neutralizing antibody while *Ivar* responded more slowly Subsequently boosted against with P IF and adjuvant, Ivar also responded with a rapid increase in neutralizing antibody

Fig 5 shows that, once established, high levels of circulating antibody can be maintained for several months, suggesting that there is continuous antibody production. Later injections of *P IF with adjuvant* elicited even higher levels of neutralizing antibody (up to 1 1,200 000) showing that the immune response to interferon could still be stimulated.

DISCUSSION

Purification seems to enhance the immunogenicity of human leukocyte interferon. It is possible that the antigenic properties of leukocyte interferon are modified by the purification procedure. Human leukocyte interferon can survive the effects of many denaturants, without loss of biological activity (*Mogensen & Cantell* 1973 *Edy et al.* 1974 *Cantell et al.* 1974) It is thus possible that purification could alter the antigenic nature of human leukocyte interferon, to the extent that it would raise antibodies even in poor responders. This matter is of some clinical importance. Alternatively the reduction of contaminating proteins may improve the response of the animal to interferon.

Some of the guinea pigs responded to less than 0.5 million units of interferon, by producing antibodies. The specific activities of pure interferons may be found to exceed 10^9 units/mg protein, if and when complete purification is achieved (*Ng & Vilcek* 1972) If such is the case, it means that guinea pigs have responded to less than 1.0 μg of interferon protein. Whether the high neutralizing titres obtained reflect exceptional immunogenicity on the part of interferon molecule, or merely the sensitivity of the biological assay for interferon is not clear

Anti-interferon globulins may prove a useful research tool in the future. A more immediate use is the preparation of high titred antisera for the purification of interferon by affinity chromatography (*Sipe et al.* 1973 *Anfinsen et al.* 1974 *Berg et al.* 1975) This study shows that suitable schedules will give antisera in sheep and rabbits, with neutralizing titres of more than 1 100,000 The binding capacity of anti-interferon globulins, immobilized on agarose, appears to exceed their capacity to neutralize human leukocyte interferon (unpublished observation) Thus, 1,000 ml of sheep serum with a neutralizing titre of 1 100,000, would have a theoretical binding capacity in excess of 100 million units of interferon.

Although the schedules described in this paper are not necessarily the most efficient, it is possible to estimate the type of schedule that will prove most effective. We suggest the following several weekly injections of interferon without adjuvant, followed by a rest when the anti-interferon titres start to rise later a booster (or two) of high-titred interferon admixed with Freund's complete adjuvant. By using interferon preparations of different purity (*Berg et al.* 1975) or different origin (*Anfinsen et al.* 1974) for the "priming" and the boosting doses, it may be possible to reduce responses to the numerous impurities present in interferon preparations, at a time when the response to interferon itself is at a peak.

Following the outline suggested above with a potent booster injection of partially purified mouse interferon (*Tovey et al.* 1974) and Freund's complete adjuvant, the antimouse interferon activity in the serum of an immunized sheep was raised from 1 6,000 to over 1 1,000,000 within 3 weeks (*M G Tovey & D Brouty-Boye* Personal communication)

REFERENCES

Anfinsen C B., Bose S., Corley L. & Gurari-Rotman D: Partial purification of human interferon by affinity chromatography Proc. nat. Acad. Sci. (Wash.) *71* 3139–3142, 1974

Berg, K., Ogburn C A., Paucker K., Mogensen K. E. & Cantell, K Affinity chromatography of human interferon. J Immunol. *114* 640–644 1975.

Cantell K & Pyhälä L. Circulating interferon in rabbits after administration of human interferon by different routes. J gen. Virol. *20* 97–104 1973.

Cantell K Hirvonen S., Mogensen, K. E. & Pyhälä L. Human leukocyte interferon, production, purification, stability and animal experiments. In: Waymouth, C. (Ed.) The Production and Use of Interferon for the Treatment and Prevention of Human Virus Infections; Proceedings of a Tissue Culture Association Workshop, 1973. *In Vitro*: monograph No 3 Rockville, Md.: Tissue Culture Association, 1974 pp. 35–38.

Cohn E. J., Strong, L. E., Hughes W L., Mulford D J., Ashworth J N., Melin, M & Taylor H L.: Preparation and properties of

serum and plasma proteins IV A system for the separation into fractions of the protein and lipoprotein components of biological tissues and fluids. J Amer chem. Soc. *68* 459–475 1946.

Duc-Goiran P Galliot B & Chany C., Studies on virus-induced interferons produced by the human amniotic membrane and white blood cells. Arch. ges. Virusforsch. *34* 232–243 1971

Edy V., Billiau A. & DeSomer P., Stabilization of mouse and human interferons by acid pH against inactivation due to shaking and guanidine hydrochloride. Proc. Soc. exp. Biol. (N Y) *146* 249–253 1974

Gresser I., Bandu M T., Brouty-Boye D & Tovey M G.: Pronounced antiviral activity of human interferon on bovine and porcine cells. Nature (Lond.) *251* 543–545 1974

International Symposium on Standardization of Interferon and Interferon Inducers London (1969) *Symposia Series in Immunological Standardization 14* 326. Basel and New York Karger

Levy-Koenig, R. E. Mundy M J & Paucker K. Immunology of Interferons. I. Immune response to protective and non-protective interferons. J Immunol. *104* 785–790 1970 a.

Levy-Koenig, R. E. Golgher R. R. & Paucker K., Immunology of Interferons. II. Heterospecific activities of human interferons and their neutralization by antibody J Immunol. *104* 791 797 1970 b.

Mogensen K. E. & Cantell, K.: Stability of human leukocyte interferon towards heat. Acta path. microbiol. scand. Sect. B, *81* 382–383, 1973.

Mogensen K E. & Cantell K.: Loss of specific antigenicity on carboxymethylation of mercaptoethanol-reduced interferon of human leukocyte origin. Intervirology *2* 5 –55 1974

Ng, M H & Vilcek J., Interferons. Advanc. Protein Chem. *26* 173–242, 1972.

Ogburn C A., Berg, K & Paucker K., Affinity chromatography of interferon. J Immunol. *111* 1206–1218, 1973

Sipe J D., DeMaeyer-Guignard, J Fauconnier B. & DeMaeyer E. Purification of mouse interferon by affinity chromatography on a solid-phase immunosorbent. Proc. nat. Acad. Sci. (Wash.) *70* 1037–1040 1973

Strander H & Cantell K Production of interferon by human leukocytes *in vitro* Ann. Med. exp. Fenn. *44* 265–273 1966.

Tovey M G., Begon-Lours, J & Gresser I A method for the large scale production of potent interferon preparations. Proc. Soc. exp. Biol. (N Y) *146* 809–815 1974

Acta path. microbiol. scand. Sect. B, 83 451–456, 1975

HUMAN SERUM ANTIBODIES AGAINST HEAT-STABLE ANTIGENS FROM *YERSINIA ENTEROCOLITICA*

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Mæland, J A. & Digranes, A. Human serum antibodies against heat-stable antigens from *Yersinia enterocolitica*. Acta path. microbiol. scand. Sect. B, 83 451–456 1975

Human sera, 200 from blood donors and five from patients with infection caused by *Yersinia enterocolitica* serotype 3 (Y.e. 3) were tested by indirect haemagglutination for antibodies against antigens from Y.e. serotypes 3 and 9 Erythrocytes were sensitized using extracts prepared by heating of the bacteria at 100° C for 60 min. All sera tested showed antibody activity against antigen from both Y.e. serotypes. In blood donors, the titres ranged from 4 to 512, and in patients from 512 to 2048 against the Y.e. 3 extract, from 32 to 256 against the Y.e 9 extract. The results of inhibition and absorption experiments showed that human antibodies against the common enterobacterial antigen (CA) agglutinated the sensitized erythrocytes and that anti-CA antibody was present in all sera tested. Some blood donor sera and all the patient sera also contained antibodies that could not be inhibited by CA when tested against antigen in the Y.e. 3 extract. Only these sera had the ability to agglutinate heat-treated Y.e. 3 bacteria. Presumably these antibodies were directed against the O antigen of the Y.e. 3 strain.

Key words *Yersinia enterocolitica* human serum antibodies heat-stable antigens.

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Since the appearance of the report by Carlsson *et al* (4) in 1964 on an infection caused by *Yersinia enterocolitica* (Y.e.) increasing attention has been paid to this microorganism. By now Y.e. is classified as a member of the *Enterobacteriaceae* (5) This is consistent with recent reports (10, 14) indicating production of the common enterobacterial antigen by Y.e. strains. Different O-serotypes of Y.e. have been described by *Winblad* (24 26) In Scandinavia, the majority of Y.e. infections in man are caused by serotypes 3 or 9 (1 8, 23) type 3 being predominant in Norway (9 20) Isolation of the pathogen from clinical specimens is most reliable in the diagnosis of infection. However testing of serum for antibodies against Y.e. antigens is also diagnostically useful.

Antibody against the O-antigen of intestinal bacilli may be detected by indirect haemagglutination using erythrocytes sensitized with bacterial heat extracts (15 16 19) Although the same technique has been used to detect human antibodies against Y.e. antigens (11) the potential usefulness of this method in the diagnosis of Y.e. infections has not been fully elucidated. This report presents the results obtained by testing sera from blood donors and patients with Y.e. infection

against erythrocytes sensitized with heat extract from Y. e. serotypes 3 and 9

MATERIALS AND METHODS

Sera

Human sera, 200 from blood donors and 5 from patients with infections caused by Y.e. serotype 3 were examined. The diagnosis of Y.e. infection was verified bacteriologically. All sera were stored at −20 C and heated at 56 C for 30 min before testing.

Bacterial Strains

Y. enterocolitica serotypes 3 and 9 (Y.e. 3, Y.e. 9) were received by courtesy of Professor *Winblad* Malmö, Sweden. *Salmonella typhimurium* was obtained from the State Serum Institute, Copenhagen, Denmark. The bacteria were kept lyophilized.

Culture

All strains were cultivated on supplemented agar plates as reported (14). The bacteria were harvested in saline, collected by centrifugation at 6,000 × *g* for 20 min and kept at −20° C.

Preparation of Bacterial Extracts

Heat extracts (100° C, 60 min) were prepared from the Y.e. strains as before (14). The common enterobacterial antigen (CA) was prepared from *S. typhimurium* (14) using the technique described by *Suzuki et al.* (21). The heat extracts and the CA extract were dialysed and lyophilized.

Serological Methods

Sensitization of erythrocytes. Human O Rh-negative erythrocytes were washed and packed. Sensitization of the erythrocytes with the Y.e. heat extracts or CA was carried out according to techniques described (13). The sensitizing activity of the preparations and the amounts to be used for sensitization were determined as reported (14). In the experiments, 1 mg of each preparation was used for the sensitization of 1 ml of an 1.5 per cent suspension of erythrocytes.

Indirect haemagglutination test. Twofold dilutions of serum were prepared in 0.025 ml volumes of PBS using the microtitre equipment (Flow Laboratories, Irvine, Scotland) and 0.025 ml of the suspension of sensitized erythrocytes (0.75 per cent) was added. The plates were incubated at 4 C for 20 h. The agglutination was read by observing the pattern formed by the sedimented erythrocytes. The titre was defined as reported (14). Sensitized erythrocytes in PBS and serum mixed with unsensitized erythrocytes served as controls.

Absorption of serum. Serum, 0.1 ml, and 2 mg of the Y.e. extract in 0.3 ml PBS were mixed, incubated at 4 C for 20 h and centrifuged at 1,000 × *g* for 20 min. The *supernatant was tested for* antibody activity.

Test for inhibition of indirect haemagglutination by CA extract. Twofold dilutions of serum were prepared and an equal volume of the CA extract (2 mg/ml) was added. PBS added to diluted serum served as control. The mixtures were incubated at 20 C for 30 min. Sensitized erythrocytes were then added and the agglutination recorded as described above.

Bacterial agglutination test. Y.e. 3 and Y.e. 9 bacteria were autoclaved, washed, suspended in PBS and standardized to give an optical density of 0.50 at 540 nm. Twofold dilutions of the sera were prepared in tubes in 0.2 ml volumes of PBS and 0.2 ml of the antigen was added. The tubes were incubated at 52 C overnight. Titre of serum is defined as the reciprocal of the highest serum dilution giving visible agglutination of the bacteria.

RESULTS

Titres of Human Sera in Indirect Haemagglutination Test

Sera from 200 blood donors and from five patients with Y.e. infection were tested against erythrocytes sensitized with heat extract from Y.e. 3 or Y.e. 9 bacteria. Antibody activity was detected in all sera examined (Table 1). In blood donors, the titres of antibody against the Y.e. 3 antigen ranged from 4 to 512 and against the Y.e. 9 from 4 to 256. In tests with Y.e. 3 and Y.e. 9 extracts, the titres most frequently shown by blood donor sera were 32 and 16, respectively. The sera either showed concordant titres in tests with antigen from the two strains or higher titres against antigen from Y.e. 3.

Sera from patients showed higher titres than those from blood donors against erythrocytes sensitized with extract from Y.e. 3. Increased titres of antibody against the Y.e. 9 antigen were less evident.

Effect of Absorption of Sera with the Y.e. Extracts

All sera from patients and seven sera selected randomly from the blood donors were

TABLE 1 *Titres in Indirect Haemagglutination Test of Human Sera Tested against Erythrocytes Sensitized with Heat Extract from* Yersinia Enterocolitica *(Y.e.) Serotype 3 or 9*

Serum from	Antigen from	No. of sera with titre <4	4-8	16-32	64 128	256-512	1024-2048	No. of sera tested
Blood donors	Y.e. 3	0	14	103	73	10	0	200
	Y.e. 9	0	37	123	38	2	0	200
Patients infected	Y.e. 3					1	4	5
with Y.e. 3	Y.e. 9			1	2			5

absorbed with Y.e. extract and then tested for antibody activity. Using five of the blood donor sera, antibody against erythrocytes sensitized with the Y.e. 3 or Y.e. 9 extract was removed by absorption with either of the extracts. The results obtained by absorption and testing of sera no. 51 and 199 from blood donor and patient sera are shown in Table 2. Using these sera, antibody against erythrocytes sensitized with Y.e. 3 extract could be removed by absorption with the same extract, but not with the Y.e. 9 extract. On the other hand, either extract depleted the sera of activity against antigen in the Y.e. 9 extract, indicating the presence of antibody against a common antigen. Further experiments were designed to test the hypothesis that antibodies against the common enterobacterial antigen were involved.

Test for Inhibition of Antibody Activity by the Common Enterobacterial Antigen (CA)

The test was carried out by adding CA extract to twofold dilutions of serum before the sensitized erythrocytes were added. In the control, CA was replaced by PBS. The patient sera and 30 of the sera from blood donors were tested for inhibition of antibody activity by CA and for activity in the bacterial agglutination test.

It applies to all sera tested that the CA extract reduced the activity against the Y.e. 9 antigen to titres <4. These sera did not show activity in the bacterial agglutination test with the Y.e. 9 strain.

When erythrocytes sensitized with the Y.e. 3 extract were applied, the CA extract inhibited all antibody activity (titre <4) in serum from 23 of the blood donors. These sera did not show agglutination of Y.e. 3 bacteria. As regards the remaining 7 sera from blood donors antibody activity against erythrocytes sensitized with the Y.e. 3 extract was not, or only partly inhibited by the CA extract (Table 3). Furthermore, four of these sera agglutinated heat-treated Y.e. 3 bacteria. Antibodies in the patient sera against the sensitizing antigen in the Y.e. 3 extract were not inhibited by CA. All of these sera ag

TABLE 2 *Sera from Blood Donors and Patients Tested by Indirect Haemagglutination against Antigen in Heat Extract from Y.e. 3 or Y.e. 9. Titres before and after Absorption with Heat Extract are Shown*

Treatment of serum	Antigen from	Blood donor No. 51	199	Patient No. 1	2	3	4	5
None	Y.e. 3	128	512	2048	2048	1024	1024	512
Abs. Y.e. 3	Y.e. 3	<4	<4	<4	<4	<4	<4	<4
Abs. Y.e. 9	Y.e. 3	128	32	2048	2048	1024	1024	512
None	Y.e. 9	32	256	64	128	32	256	256
Abs. Y.e. 3	Y.e. 9	<4	<4	<4	<4	<4	<4	<4
Abs. Y.e. 9	Y.e. 9	<4	<4	<4	<4	<4	<4	<4

TABLE 3 *Titres of Sera from Blood Donors and Patients in Indirect Haemagglutination and Bacterial Agglutination Tests Using Erythrocytes Sensitized with Heat Extract from Y.e. 3 and Heat Treated Y.e. 3 Bacteria Respectively. Titres in Indirect Haemagglutination Test with PBS or CA Extract Added to Serum are Shown*

Serum from		Indirect haemagglutination		Bacterial agglutination
		Serum + PBS	Serum + CA	
Blood donor No.	51	128	128	64
	119	512	64	16
	122	128	64	16
	126	128	16	<4
	140	256	64	64
	161	128	16	<4
	189	256	16	<4
Patient No.	1	2048	2048	1024
	2	2048	2048	1024
	3	1024	1024	256
	4	1024	1024	1024
	5	512	512	128

glutinated heat treated Y.e. 3 bacteria (Table 3) Inhibition by CA did not affect the titre in the bacterial agglutination test of any of the sera examined.

DISCUSSION

All of 200 sera from blood donors showed antibody activity against erythrocytes sensitized with heat extract from the Y.e. 3 or Y.e. 9 bacteria with titres ranging from 4 to 512 The frequency of Y e. infection in the local population is unknown. However it would seem improbable that this microorganism has stimulated antibody production in all individuals examined. The results obtained could be explained by assuming that antibodies against the common enterobacterial antigen (CA) were present in the sera and that these antibodies, in a manner similar to that of rabbit anti-CA antibodies (14) agglutinated erythrocytes sensitized with Y e. heat extracts. In all human sera tested for inhibition, antibody activity against antigen in the Y.e. 9 extract was neutralized by CA. The majority of the sera were also inhibited by this antigen when tested against the Y.e. 3 extract. These results are interpreted to the effect that human anti-CA antibodies have the ability to agglutinate erythrocytes sensitized with the Y.e. heat extracts, and that these antibodies were present in all sera tested. This interpretation accords with the observation that normal human serum contain antibodies to CA (17 22)

Haemagglutinating antibodies in human serum against heat-stable Y.e. antigens were recently described by *Lysy & Knapp* (11) However the possibility that the antibodies detected could be directed against CA was not considered. The range of titres shown by the blood donor sera tested in this study was equivalent to titres obtained by the indirect haemagglutination test of sera from healthy subjects, using heat extracts from various *Enterobacteriaceae* (6, 18, 23) The activity has variously been ascribed to "natural antibodies" (12) or antibodies against the O-antigen of the bacterium (23) According to the results of our study anti-CA antibodies may have been involved.

It has been shown that antibody to CA promotes phagocytosis by polymorphonuclear leucocytes of enteric bacilli (5) The question whether this antibody has the ability to enhance the resistance of human beings to infection by Y.e deserves attention.

The activity of the patient sera against antigen in the Y.e. 3 extract was not affected by inhibition by CA or absorption with Y.e. 9 extract. This finding is compatible with the presence of antibodies against an antigen possessed only by Y.e. 3, presumably the O antigen. This accords with proven Y.e infection in the patients, and with high titres of the sera in the bacterial agglutination test using heat-treated Y.e. bacteria. Anti-CA antibodies do not have the ability to agglutinate bacteria (5 7) which explains why the Y.e. 9 strain was not agglutinated by any of the sera tested. Antibodies showing specificity for the O antigen of Y.e. 3 were also present in some of the blood donor sera. This was demonstrated by absorption and inhibition experiments and by the bacterial agglutination test. Whether these antibodies, being present in comparatively low titres, were acquired during present or past infection with Y.e. 3 is not known. However this possibility would accord with the predominance of serotype 3 among Y.e. isolated in this community. Another explanation may be that these antibodies were produced in response to stimulation by cross-reacting bacteria. Cross-reactivity between Y.e. strains and other bacterial species has been described (2, 25 26).

According to our view the indirect haemagglutination test using heat extracts from Y.e. strains may be useful in the diagnosis of infection. At present, however the test procedure is hampered by the necessity of neutralizing antibody to CA.

REFERENCES

1. *Ahvonen, P.:* Human yersiniosis in Finland. 1 Bacteriology and Serology. Ann. Clin. Res. *4:* 30–38, 1972.
2. *Ahvonen, P. Jansson Elli & Aho K.* Marked cross-agglutination between *Brucella* and a subtype of *Yersinia enterocolitica*. Acta path. microbiol. scand. 75 291–295, 1969
3. *Bergey's manual of determinative bacteriology* 8. ed. The Williams & Wilkins Company Baltimore, 1974.
4. *Carlsson M. G., Ryd H & Sternby N* A case of human infection with *Pasteurella pseudotuberculosis* X. Acta path. microbiol. scand. 62 128–132, 1964
5. *Domingue G J & Neter E.*. Opsonizing and bactericidal activity of antibodies against common antigen of *Enterobacteriaceae* J Bact. *91* 129–133 1966.
6. *Kunin C. M.:* Antibody distribution against nonenteropathic *E. coli*. Relation to age, sex, and breast feeding. Arch. intern. Med. *110* 182–192, 1962.
7. *Kunin C M Beard Mary V & Halmagyi, Norma E.:* Evidence for a common hapten associated with endotoxin fractions of *E. coli* and other *Enterobacteriaceae* Proc. Soc. exp. Biol. (N Y) *111* 160–166 1962.
8. *Larsen J Hannover* Human yersiniose. Resultatet af serologisk undersøgelse for infektion med *Yersinia enterocolitica*. Ugeskr Laeg. *134* 431–436, 1972.
9. *Larsen, J & Bakkhus O : Yersinia enterocolitica*-infeksjoner hos mennesker. T norske Laegeforen. *91* 635–640 1971
10. *Le Minor L. Chalon A.-M & Véron M* Recherches sur la présence de l'antigène commun des *Enterobacteriaceae* (antigène Kunin) chez les *Yersinia Levinea, Aeromonas* et *Vibrio* Ann. Inst. Pasteur *123:* 761–774 1972.
11. *Lysy J & Knapp W.*. Serological studies with *Y enterocolitica*. Contributions to Microbiology and Immunology vol. 2. Yersinia, Pasteurella and Francisella, Karger Basel 1973 pp 42–53.
12. *Michael, J G & Rosen F S.:* Association of "natural" antibodies to Gram-negative bacteria with the γ-macroglobulins. J exp. Med. *118* 619–626 1963
13. *Maeland J A :* Antibodies in human sera against antigens in gonococci, demonstrated by a passive haemolysis test. Acta path. microbiol. scand. 67 102–110, 1966.
14. *Maeland, J A & Digranes A* Common enterobacterial antigen in *Yersinia enterocolitica*. Acta path. microbiol. scand. Sect. B, *83:* 382 386, 1975.
15. *Needell, M H., Neter E., Staubitz W J & Bingham M.:* The antibody (hemagglutinin) response of patients with infections of the urinary tract. J Urol. (Baltimore) *74:* 674–682, 1955.
16. *Neter E., Bertram L. F., Zak D A., Murdock M R. & Arbesman C. E.*. Studies on hemagglutination and hemolysis by *Escherichia coli* antisera. J exp. Med. *96* 1–15, 1952.
17. *Neter E., Kennedy E. A & Jewett T C.*. Antibody response to common enterobacterial antigen of children with pyogenic peritonitis. Infection *1* 12–16, 1973
18. *Neter E., Westphal O., Lüderitz O., Gino R. M & Gorzynski, E. A.:* Demonstration of antibodies against enteropathogenic *Escherichia coli* in sera of children of various ages. Pediatrics *16* 801–808, 1955.

19 *Neter E. Westphal, O Lüderitz, O & Gorzynski, E. A* The bacterial hemagglutination test for the demonstration of antibodies to *Enterobacteriaceae* Ann. N Y Acad. Sci. *66* 141–156, 1956.

20 *Osnes M & Larsen, J* Artritter og polymyalgier ved *Yersinia enterocolitica* infeksjoner T norske Laegeforen. *94* 225–228, 1974

21 *Suzuki T Gorzynski E. A. & Neter E.* Separation by ethanol of common and somatic antigens of *Enterobacteriaceae* J Bact. *88* 1240–1243 1964

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25 *Winblad S.* Studies on O-antigen factors of *Yersinia enterocolitica*" Int. Symp. on Pseudotuberculosis. Symposia Series. Progr immunobiol. Standard. vol. 9. Karger Basel 1968, pp. 337–342.

26. *Winblad S.* Studies on the O-serotypes of *Yersinia enterocolitica*. Contributions to Microbiology and Immunology vol. 2. Yersinia, Pasteurella and Francisella, Karger Basel 1973 pp. 27–37

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CANINE MYCOPLASMAS CULTURAL AND BIOCHEMICAL STUDIES OF TYPE AND REFERENCE STRAINS

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Media and conditions for cultivation of type and reference strains of the mycoplasmas presently known (except *M bovigenitalium A. laidlawii* and *Ureaplasma* strains) to occur in dogs are described. The colonial morphology ultrastructure, filterability and absence of reversion confirmed that the strains belong to the order *Mycoplasmatales*. All strains were cholesterol dependent and therefore of the family *Mycoplasmataceae* genus *Mycoplasma*. Finally the most useful biochemical characteristics are specified.

Key words Canine mycoplasmas; cultural, biochemical studies.

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In 1972 the Subcommittee on the Taxonomy of Mycoplasmatales published a proposal for minimal requirements for description and classification of new species of the order *Mycoplasmatales* (19) class *Mollicutes*

According to these requirements, it should be shown that organisms classified as *Mycoplasmatales* lacked a cell-wall, possessed the typical colonial morphology (likened to a fried-egg) were filterable through membrane filters of pore size 450 nm, and they should not revert to bacteria when cultivated on media without inhibiting substances. The last mentioned criterion is set up to exclude L-phase variants.

Mycoplasmatales is subdivided into two families, *Mycoplasmataceae* and *Acholeplasmataceae* Members of the former family require sterol for growth whereas this is not required by the latter (7)

In the family *Mycoplasmataceae* two genera are recognized so far *Mycoplasma* and *Ureaplasma*. Organisms of the genus *Ureaplasma* (18) split urea, whereas those of genus *Mycoplasma* do not. In the family *Acholeplasmataceae* one genus is established viz. *Acholeplasma*.

In the genus *Ureaplasma* only one species is recognized. This species is named *U urealyticum* (18) and includes all human strains which catabolize urea irrespective of serological differences between the strains. In contrast, the classification of species into the genera *Mycoplasma* and *Acholeplasma* is primarily based on serological characteristics. Thus, before a new species is established, it would be ideal if it was serologically com

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TABLE 1 *Type or Reference Strains of Mycoplasma Species Occurring in Dogs*

Species or serogroup	Type or reference strain	Isolated by	Source
M. spumans	PG13	*D. G. ff. Edward & W. A. Fitzgerald* (6)	Vagina
M. maculosum	PG15	*D. G. ff. Edward & W. A. Fitzgerald* (6)	?
Serogroup D	MH5408	*D. Armstrong et al.* (2)	Throat
M. gateae	CS	*Cole et al.* (4)	Saliva (cat)
M. canis	PG14	*D. G. ff. Edward & W. A. Fitzgerald* (6)	Throat
M. edwardii	PG24	*D. G. ff. Edward & W. A. Fitzgerald* (6)	Throat
M. molare	H542	*S. Rosendal* (15)	Throat
M. cynos	H831	*S. Rosendal* (14)	Lung
M. sp.	HRC689	*M. F. Barile et al.* (3)	Larynx
M. feliminutum	BEN	*Heyward et al.* (12)	Mouth (cat)
M. bovigenitalium	PG11	*D. G. ff. Edward* (5)	Genital tract (cow)
A. laidlawii	PG8	*Laidlaw & Elford* (13)	Sewage

the above experiments. The first set was incubated at 37 C, the second at 27 C and the third at 22° C.

3. *Filtration Experiments*

A culture of each strain was examined for filterability through Millipore filters (Bedford, Mass. U.S.A.) of 450 nm, 200 nm, 100 nm, and 50 nm pore sizes (14). The filters were placed in Swinnex-25 Millipore filter adapters.

4. *Morphological Studies*

Colonies of organisms cultivated under optimal conditions were examined by means of a stereomicroscope. The ultrastructure was studied by electron microscopy these observations will be published in greater detail in a subsequent paper.

5. *Absence of Reversion to Bacteria*

All strains were grown under optimal conditions in fluid and solid media without antibacterial agents. The strains were subcultured 5 times. At each subcultivation, 0.01 ml of culture was streaked on plates. The colony morphology was studied both macroscopically and microscopically The microscopical examination was performed according to the description given by *Freundt* (10)

6a. *Cholesterol Requirement*

The strains were examined for cholesterol requirement by the method described by *Edward* (8) A suspension in PBS pH 7 4 of each strain was diluted in serial 10-fold dilutions in PBS pH 7 4 Three sets of plates from each dilution were inoculated. A Serum free plates, B A plates to which palmitic acid and bovine serum albumin were added, and C B plates to which cholesterol was added. The exact composition of A, B and C plates has been described by *Erno & Stipkovits* (9) The inoculated plates were incubated under optimal atmospheric conditions and examined for growth every second day for 10 days.

6b. *Sensitivity to Digitonin*

Cholesterol dependence is determined indirectly by the test as strains dependent on cholesterol are very sensitive to digitonin whereas non-requiring strains are by far less sensitive or resistant. Filter paper discs containing 0.5 mg digitonin were placed on plates inoculated with a culture of approximately 10^6 c.f.u./ml. The zone of inhibition was measured after 4 days of incubation under optimal conditions (11)

7 *Biochemical Studies*

All strains were examined for glucose and arginine catabolism, phosphatase activity production of film and spots, and haemolysis of guinea pig erythrocytes. Media and test procedures have been described earlier (16)

RESULTS

Media experiments. The media providing the best growth of the type and reference strains are shown in Table 2. B medium was found to be suitable for most strains. Only PG13 PG15 and MH5408 grew better on N medium. However the B medium may be used for these strains as well, although their growth is delayed and does not always the same level as in the N

TABLE 2. *Results of Tests of Media and of Experiments in which Growth at Different Temperatures Was Studied*

	Media experiments			Temperature experiments			
	Preferred medium	Time of incubation required for maximal growth in fluid culture	Maximal c.f.u./ml in medium preferred	37		27	
				M	T	M	T
M. spumans PG13	N	2 days	10^9	6.0	5×10^8	4.4	4×10^5
M. maculosum PG15	N	2 days	6×10^7	9.0	4×10^8	6.8	10^6
MH5408	N	2 days	10^8	4.0	5×10^5	2.6	3×10^7
M. gateae CS	B	2 days	3×10^7	8.0	2×10^7	4.8	10^8
M. canis PG14	B	2 days	10^8	6.0	10^8	3.6	8×10^5
M. edwardii PG24	B–N	2 days	2×10	5.0	10^8	5.0	10^8
M. molare H542	B	1 day	10^7	5.0	6×10^8	3.6	10^4
M. cynos H831	B†	1 day	10^7	6.4	10^8	4.4	10^8
HRC689	B†	2 days	2×10^7	8.8	10^8	1.6	10^4
M. feliminutum BEN	B†	1 day	2×10^8	6.0	6×10^3	3.6	10^8

* B and N media were found to be equally good.
† N medium gave very poor grwth of these strains.
M Mean values for growth (9)
T Titre, viz. maximal c.f.u. per ml.

Gaseous requirements. All strains grew well under all 4 atmospheric conditions tested. It applies to a few strains that only slight differences were noticed. PG13 seemed to prefer aerobic incubation, PG15 and HRC689 showed a preference for N_2 or $N_2 + CO_2$, and incubation of BEN in $N_2 + CO_2$ promoted the best growth of this strain.

Growth at different temperatures. The results obtained in experiments in which growth temperature were studied are summarized in Table 2, where the mean values (M) for growth are listed as well as the maximal numbers of colonies per ml to be found after incubation of the plates at 37 C and 27 C, respectively. None of the strains were able to grow at 22 C. Whereas PG24 grew equally well at 27 C and 37 C, the growth of the remaining strains was considerably reduced at 27 C.

Filtration experiments. All strains were filterable through the 450 nm filter and strains PG15, H542, HRC689 and CS also passed 200 nm filters. They were all retained by 100 nm pores.

Morphology. The colonies of all the strains had a typical fried-egg appearance.

By electron microscopy all strains were seen to be surrounded by a triple-layered unit membrane and to lack a cell-wall. In addition, the cells were highly pleomorphic.

Absence of bacterial reversion. The strains retained their "fried-egg like" colonial morphology during 5 consecutive subcultivations in substrate without bacterial inhibitors. The microscopical examination did not reveal any bacteria like cells.

Cholesterol requirement. It has been shown earlier by Edward's method that *M. cynos* (H831) and *M. molare* (H542) require cholesterol for growth (14, 15). The remaining strains were tested in this study and they all required cholesterol.

In the case of BEN and CS, primary inoculation of A, B and C plates gave good growth on C plates (cholesterol containing) and poor growth on B plates (serum-free but with palmitic acid and bovine serum albumin). Colonies from these B plates were subcultured to C and B plates. After two

TABLE 3 *Biochemical Properties of Type and Reference Strains*

	Glucose fermentation	Arginine deamination	Phosphatase production	Film and spots (Egg Yolk medium)	Haemolysis of guinea pig erythrocytes
M spumans PG13	—	+	+	—	—
M maculosum PG15	—	+	+	+	—
MH5408	—	+	+	+	+
M gateae CS	—	+	—	—	—
M canis PG14	+	—	—	—	+
M edwardii PG24	+	—	—	—	+
M molare H542	+	—	—	+	+
M cynos H831	+	—	+	+	+
HRC689	+*	—	+*	+*	+*
M felimnitum BEN	—	—	—*	+*	—

These biochemical data have not been published before.

passages, growth appeared on C plates only.

Sensitivity to digitonin. All strains were found highly sensitive to digitonin, the zone of inhibition varying between 5.5 mm and 8.0 mm.

Biochemical properties. The biochemical properties of the type and reference strains are shown in Table 3. In the case of PG13, PG14, PG15, PG24, H831 and H542, these properties have been reported earlier (16).

DISCUSSION

Host relationship. The term "canine mycoplasma species" should be applied to species isolated from dogs only or isolated frequently from dogs and seldom from other hosts as well. The following species seem to fulfil this criterion: *M spumans*, *M canis*, *M maculosum*, *M edwardii*, *M cynos* and *M molare*. Serogroup D has been identified 9 times in isolates from dogs (ref. 2 and unpublished observations) for which reason the dog may be considered the normal habitat. The species *M gateae* which occurs frequently in cats and dogs may be regarded as a feline as well as a canine species. Definition of the normal habitat of *M feliminutum* and organisms related to strain HRC689 must await the results of further investigations. *M bovigenitalium* is a bovine species which in recent time frequently has been identified in isolates from the genital tract of dogs (unpublished observations). Together with the two previously reported isolates (16) it may be designed a canine species as well. *A. laidlawii* has been isolated from a wide spectrum of different hosts including dogs (17) and thus has no specific habitat.

Cultural characteristics. The results of experiments with media lead to the conclusion that the B medium may suffice for cultivation of all the strains. The various gaseous conditions caused negligible differences in growth and thus, for most purposes it is sufficient to use aerobic incubation. It applies to all strains that optimal growth is obtained at 37 C.

Classification into order. The filterability through a 450 nm pore size filter, the colony morphology and the electron microscopical characteristics of all strains are in agreement with the requirements for micro-organisms placed in the order *Mycoplasmatales*. None of the strains reverted to bacteria during cultivation on substrate without inhibitors.

Classification into family. The results of direct determination of the cholesterol requirement and the sensitivity of all strains to digitonin confirm the correctness of their classification into the family *Mycoplasmataceae*.

Classification into genus. As no others but urea negative mycoplasmas were included in

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M. molare H542	B	1 day	10^7	5.0	6×10^8	3.6	10^7
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CANINE MYCOPLASMAS SEROLOGICAL STUDIES OF TYPE AND REFERENCE STRAINS, WITH A PROPOSAL FOR THE NEW SPECIES, *MYCOPLASMA OPALESCENS*

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The type and reference strains of all *Mycoplasma* species or serogroups presently known to occur in dogs were compared serologically. Tests of growth inhibition and metabolism inhibition as well as indirect immunofluorescence tests did not disclose any cross reactions. Examination by double immunodiffusion revealed at least one antigen specific for each strain tested. In addition, the common antigens were found within the glucose fermenting group and within the arginine metabolizing group of *Mycoplasma* strains, respectively. No antigens were shared by these groups. The arginine positive strain MH5408, serogroup D was found to differ serologically from all other arginine positive *Mycoplasma* species. Serogroup D is therefore a new species for which the name *Mycoplasma opalescens* is proposed. The type strain is MH5408 (ATCC 27921 and NTCC 10149). Finally, serological data are presented which relate strain HRC689 to *M. mycoides*.

Key words: Canine mycoplasmas; serology; *Mycoplasma opalescens*.

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The cultural and biochemical properties of type and reference strains of mycoplasma species and serogroups occurring in dogs have been described in a previous publication (12). The type strains of *M. bovigenitalium* (PG11) and *A. laidlawii* (PG8) were not included as they had been examined in a study on bovine mycoplasmas (5). Neither were organisms of the genus *Ureaplasma* included. The classification of all strains examined as members of the family *Mycoplasmataceae*, genus *Mycoplasma* was confirmed.

Species classification must be based on serological studies. For this purpose, the Subcommittee on the Taxonomy of Mycoplasmatales (13) recommends tests of the disc growth inhibition (GI) and/or metabolism inhibition (MI) together with complement fixation, double immunodiffusion, agglutination, and fluorescent antibody tests.

The present publication deals with serolo-

gical studies of type and reference strains of canine mycoplasma species and serogroups by means of growth inhibition metabolism inhibition, double immunodiffusion and indirect immunofluorescence.

MATERIALS AND METHODS

A Strains

The serological investigations were carried out with the same strains studied in the previous paper (12) together with the type strains of *M bovigenitalium* (PG11) and *A. laidlawii* (PG8) which also occur in dogs. The arginine positive strain MH5408, the reference strain of serogroup D was compared serologically with the type strains of the arginine positive species listed in Table 1 The type strains of glucose positive species (Table 2) were included and used for comparison with the glucose ferment ing strain HRC689

B. Antisera

Antisera were produced as described earlier (10 11)

C Serological Tests

1 Growth inhibition. Filter paper discs were soaked with 0.02 ml of serum, dried at 37 C, and then stored at 4 C until use. The test was per formed on C medium (6) which is similar to the B medium (5) but contains only 2.5 per cent horse serum and no yeast extract. The plates were

TABLE 1 *Strains of Arginine Positive* Mycoplasma *Species Used for Serological Comparison with Strain MH5408 (serogroup D Armstrong et al.) (3)*

Species	Strain
M arthritidis	PG6
M primatum	HRC292
M arginini	G230
M alkalescens	D12
M c pricolum	Calif. kid
M gallinarum	PG16
M ners	PG30
M male gridis	17529
M hyosynoviae	S16
M hominis	PG21
M orale	CH19299
M buccale	CH20247
M faucium	DC-333
M salivarium	PG20
M fermentans	PG18

TABLE 2. *Strains of Glucose Fermenting Mycoplasma Species Used for Serological Comparison with Strain HRC689 (Barile et al.) (4)*

Species	Strain
M felis	CO
M caviae	G122
M neurolyticum	Type A
M pulmonis	Ash
M gallisepticum	PG31
M anatis	1340
M hyorhinis	BTS-7
M pneumoniae	FH
M fermentans	PG18
M conjunctivae	HRC581
M ovipneumoniae	Y 98
M c pricolum	Calif. kid
M p trefaciens	KS-1
M bovirhi is	PG43
M bovoculi	M165/69
M mycoides subsp myco des	PG1
M mycoides subsp c pri	PG3
M mycoides subsp mycoides	Y-goat (2)
Group L (Al Aubaidi)	B-144P (1)
Group 7 (Leach)	PG50 (9)

inoculated by the "running drop technique with 0.01 ml of a culture containing 10^5 c.f.u./ml. After drying of the inoculum serum disc was placed in the middle of the mycoplasma streak The plates were incubated for 4 days at 37° C before reading.

Acceptable homologou inhibition was not obtained in every case by this procedure In the case of strain PG11 inhibition was found to be better if the plate cultures were incubated initially at 27 C for two days and then t 37 C for a further two days In the case of strains PG14 H831 BEN and PG8, a well, measuring 4 mm in diameter was made in the middle of the mycoplasma streak and filled w th serum it wa refilled a further tw times within 8 hours post noculation. The plates were incubated for 2 days at 27 C and then for 2 days at 37 C before read ng

2 Metabolism inhibitio a Antigens. Glucose fermenting strains were lt ted in 10 ml of BG medium until appearanc of colour change The BG medium consisted of B med um (5) with the addition of 0 5 per cent glucose and 0 003 per cent phenol red The pH was djusted to 7.8.

Arginine hydrolysing str n were cultivated in 10 ml of BA medi m unt l olour change The BA medium onsisted of B med um w th 1 per cent L-arginine and 0 003 per ent phenol red added. The pH was adjusted to 7 5

M bov genitalium PG11 glucose and arginine negati e. Although t does reduce tetrazolium under certain conditions this activity is too lo-

TABLE 1. Growth Inhibition. Antigenic Relationships of Type and Reference Strains of Mycoplasmas Occurring in Dogs

Antigen Species or serogroup	Type or reference strain	Antiserum PG13	PG15	MH 5408	C8	PG14	PG24	H542	H831	HRC 689	BEN	PG11	PG8	Incubation temperature	Days of incubation	Method for application of serum
M. spumans	PG13	4.0	0	0	0	0	0	0	0	0	0	0	0	37 C	4	D
M. maculosum	PG15	0	2.0	0	0	0	0	0	0	0	0	0	0	37 C	4	D
Serogroup D (*Armstrong et al.*) (3)	MH5408	0	0	3.5 R	0	0	0	0	0	0	0	0	0	37 C	4	D
M. gateae	C8	0	0	0	3.5	0	0	0	0	0	0	0	0	37 C	4	D
M. canis	PG14	0	0	0	0	6.0	0	0	0	0	0	0	0	27 C 37 C	2 2	W
M. edwardii	PG24	0	0	0	0	0	4.0	0	0	0	0	0	0	37 C	4	D
M. molare	H542	0	0	0	0	0	0	5.0	0	0	0	0	0	37 C	4	D
M. cynos	H831	0	0	0	0	0	2.0*	0	7.5	2.0	0	0	0	27 C 37 C	2 2	W
Unidentified (*Barile et al.*) (4)	HRC689	0	0	0	0	0	0	0	0	2.0 NT	0	0	0	37 C	4	D
M. feliminutum	BEN	0	0	0	0	0	0	0	0	0	4.0 NT	0	0	27 C 37 C	2 2	W
M. bovigenitalium	PG11	0	0	0	0	0	0	0	0	0	0	5.0	0	27 C 37 C	2 2	D
A. laidlawii	PG8	0	0	0	0	0	0	0	0	0	0	0	5.0 R	22 C 27 C	2 1	W

R The zone of inhibition was not total, but a significant reduction in size and number of colonies was seen.

NT The zone of inhibition was not total, but the number of "break through" colonies was less than 10

D Serum soaked in discs.

W: Serum filled into wells three times.

* This inhibition is regarded as unspecific, as pre-immune sera were inhibitory as well.

TABLE 4 *Metabolism Inhibition. Antigenic Relationships of Type and Reference Strains of Mycoplasmas Occurring in Dogs*

Antigen		Antiserum											
Species or serogroup	Type or reference strain	PG13	PG15	MH 5408	CS	PG14	PG24	H542	H831	HRC 689	BEN	PG11	PG8
M spumans	PG13	128	0	0	0	0	0	0	0	0	0	0	0
M maculosum	PG15	0	1024	0	0	0	0	0	0	0	0	0	0
Serogroup D (*Armstrong et al.*) (3)	MH5408	0	0	≥ 2048	4	0	0	0	0	0	0	0	0
M gateae	CS	0	0	0	≥ 2048	0	0	0	0	0	0	0	0
M canis	PG14	0	0	0	0	1024	0	0	0	0	0	0	0
M edwardii	PG24	0	0	0	0	0	512	0	0	0	0	0	0
M molare	H542	0	0	0	0	0	0	≥ 2048	0	0	0	0	0
M cynos	H831	0	0	0	0	0	0	0	512	0	0	0	0
Unidentified (*Barile et al.*) (4)	HRC689	0	0	0	0	0	0	0	0	256	0	0	0
M bovigenitalium	PG11	0	0	0	0	0	0	0	0	0	0	512	0
A laidlawii	PG8	0	0	0	0	0	0	0	0	0	0	0	1024

Titres lower than 2 are recorded as zero.

Note Titration with *M felimentum* (BEN) was not possible as this strain in both glucose arginine tetrazolium, and phosphatase negative.

consistent for the MI test. However PG11 produces phosphatase and, in order to utilize this property in the MI test, this strain was cultivated in N-medium (5) to which 0.01 per cent Na-phenolphthalein diphosphate was added. The culture was incubated until a red colour change appeared in 1-ml-samples after addition of a few drops of 5N NaOH.

The MI test could not be performed in the case of strain *M felimentum* (BEN) as the latter is glucose- arginine- phosphatase- and tetrazolium negative.

All cultures (antigens) were stored in 1-ml-aliquots at − 70 C

The antigens were titrated in order to determine the number of colour changing units (c.c.u.) The titrations were performed by means of a microtitre system (Cooke Engineering Co., Alexandria, Va.) in Disposo Trays (Linbro Chem. Co., Inc New Haven, Conn.) Two drops (0.05 ml) of 10-fold serial dilutions of the antigen plus 6 drops (0.15 ml) of the appropriate medium were mixed and incubated at 37 C. The reciprocal value of the highest dilution giving colour change indicated the number of c.c.u. per two drops (0.05 ml) of antigen. In the case of strain PG11 a colour change developed after 1 drop of 5N NaOH had been added to each well

b. *Serum titration.* Serum was inactivated at 56 C for 30 min and 2-fold dilutions were made of 1 drop (0.025 ml) of serum in 1 drop of medium. Two drops of antigen containing 100 c.c.u. and 5 drops of appropriate medium were added. The plates were read when colour change had taken place in control wells without serum corresponding to a decrease or increase in pH by 0.5 units in the case of BG and BA medium, respectively When antisera were titrated against PG11 one drop of 5N NaOH was added daily to the control wells without serum. When colour change occurred, NaOH was added to all wells and reading performed according to the subsequent colour change

3 *Double immunodiffusion* Preparation of antigens and the technical procedure used in this test have been described earlier (11) However a single modification was introduced as the mycoplasma suspension to be used for antigen preparation was frozen and thawed repeatedly to replace sonication This was done at least 10 times until no viable organisms could be demonstrated after streaking on solid medium.

Initially all antisera were tested against each of the antigens. Thereupon those sera and antigens which in the initial examination gave precipitation lines were tested in patterns suitable for further analysis of homologous and heterologous precipitation lines. Using this procedure antigen formulas applying to each strain could be established, a one precipitation line was considered to represent one antigen. The precipitating antigens were coded by lower case letters.

TABLE 5. *Double Immunodiffusion Antigenic Relationships of Type and Reference Strains of Mycoplasmas Occurring in Dogs*

Antigen		Antiserum											
Species or serogroup	Type or reference strain	PG13	PG15	MH 5408	CS	PG14	PG24	H542	H831	HRC 689	BEN	PG11	PG8
M. spumans	PG13	abc	0	0	0	0	0	0	0	0	0	0	0
M. maculosum	PG15	ab	abg	0	0	0	0	0	0	0	0	0	0
Serogroup D (*Armstrong et al.*) (3)	MH5408	ab	0	ablm	0	0	0	0	0	0	0	0	0
M. gateae	C8	ab	0	0	apq	0	0	0	0	0	0	0	0
M. canis	PG14	0	0	0	0	def	da	d	dh	0	0	0	0
M. edwardii	PG24	0	0	0	0	0	dahj	0	dh	0	0	0	0
M. molare	H542	0	0	0	0	0	dh	dhk	dh	0	0	0	0
M. cynos	H831	0	0	0	0	0	d	0	dhl	0	0	0	0
Unidentified (*Rosendal et al.*) (4)	HRC689	0	0	0	0	0	0	0	0	no	0	0	0
M. feliminutum	BEN	0	0	0	0	0	0	0	0	0	0	0	0
M. bovigenitalium	PG11	0	0	0	0	0	0	0	0	n	0	nrs	0
A. laidlawii	PG8	0	0	0	0	0	0	0	0	0	0	0	t
Antigen formulae		abc	abg	ablm	abpq	defh	dahj	dhk	dhl	no	–	nrs	t

Note: No precipitation lines were obtained with either antigen or antiserum of *M. feliminutum* (BEN)

4. *Indirect immunofluorescence* (IMF). The technique described earlier was used (10). First, the optimal dilution of each serum was determined in homologous titrations, i.e. the highest dilution giving strong fluorescence. Then, cross titrations with each serum were performed, using the 1:10 dilution as well as the optimal dilution.

5. *Serological studies.* a. *Cross examination of type and reference strains.* The 12 strains included in this part of the study were examined in double cross titrations using all 4 serological methods.

b. *Serological comparison of MH5408 with arginine positive strains.* Double cross titrations between MH5408 and the strains listed in Table 1 were performed by means of indirect IMF and GI tests. In addition, antisera against these strains were in the MI test titrated against strain MH5408.

c. *Serological comparison of HRC689 with glucose fermenting strains.* Double cross titrations between HRC689 and the strains listed in Table 2 were performed using the indirect IMF test.

RESULTS

1. Cross Examination of Type and Reference Strains

a. *Growth inhibition.* The results are shown in Table 3 together with the test procedure used for each strain. *M. cynos* (H831) was inhibited 7.5 mm by homologous antiserum and about 2 mm by antisera against PG24 and HRC689. Since, however, pre-immune sera corresponding to these antisera also inhibited H831 in a zone of 2 mm, the heterologous reactions are regarded as nonspecific. The globulin fractions (10) of these pre-immune sera and antisera were likewise inhibitory to H831.

b. *Metabolism inhibition.* The results are listed in Table 4. In general, fairly high homologous titres were obtained by this method. All heterologous titres were < 2, except for the titre of 4 found in the CS antiserum if titrated against the MH5408 antigen.

c. *Double immunodiffusion.* Antigens common to the group of glucose fermenting strains and the group of arginine hydrolyzing strains were revealed (Table 5), but no antigens were shared by the two groups. *A. laidlawii* (PG8) did not share antigens with any of the *Mycoplasma* strains. *M. bovigenitalium* (PG11), which is glucose negative, was found to possess one antigen in common with the glucose positive strain HRC689. In addition,

TABLE 6. *Indirect Immunofluorescence. Antigenic Relationships of Type and Reference Strains of Mycoplasmas Occurring in Dogs*

Antigen: Species or serogroup	Type or reference strain	Antiserum: PG13	PG15	MH 5408	CS	PG14	PG24	H542	H831	HRG 689	BEN	PG11	PG8
M spumans	PG13	+	0	0	0	0	0	0	0	0	0	0	0
M maculosum	PG15	0	+	0	0	0	0	0	0	0	0	0	0
Serogroup D (*Armstrong et al.*) (3)	MH5408	0	0	+	0	0	0	0	0	0	0	0	0
M gateae	CS	0	0	0	+	0	0	0	0	0	0	0	0
M canis	PG14	0	0	0	0	+	0	0	0	0	0	0	0
M edwardii	PG24	0	0	0	0	0	+	0	0	0	0	0	0
M molare	H542	0	0	0	0	0	0	+	0	0	0	0	0
M cynos	H831	0	0	0	0	0	0	0	+	0	0	0	0
Unidentified (*Barile et al.*) (4)	HRC689	0	0	0	0	0	0	0	0	+	0	0	0
M feliminutum	BEN	0	0	0	0	0	0	0	0	0	+	0	0
M bovigenitalium	PG11	0	0	0	0	0	0	0	0	0	0	+	0
A laidlawii	PG8	0	0	0	0	0	0	0	0	0	0	0	+

each strain was found to possess at least one specific antigen.

d. *Indirect immunofluorescence* No cross reactions were observed (Table 6)

2. Serological Comparison of Strain MH5408 with Arginine Hydrolyzing Strains

No cross reactions were observed in the double cross examinations using indirect IMF and GI In the MI test the MH5408 antigen did not react with any of the antisera against arginine positive strains (titres <2)

3 Serological Comparison of Strain HRC689 with Glucose Fermenting Strains

In the indirect IMF test, a one-way cross reaction between HRC689 and *M mycoides* subsp. *capri* (PG3) was observed. No cross reaction with any of the remaining glucose positive strains was observed. The serological relationship between strain HRC689 and *M mycoides* subsp. *capri* (PG3) and related organisms (*M mycoides* subsp. *mycoides* PG1 *M mycoides* subsp. *mycoides* Y-goat (2) the bovine group L strain B144P (1) and the bovine group 7 strain PG50 (9)) was studied further by means of GI MI and double immunodiffusion tests. One-way cross reactions were found by growth inhibition with strains Y-goat, PG3 and B144P In metabolism inhibition a one-way cross reaction was found with strains PG3 B144P and PG50 One of the antigens demonstrated in double immunodiffusion was shared by strains HRC689 PC 1 PG3 B144P and PG50.

DISCUSSION

Serological r lationships of type and reference strains. In the serological classification of mycoplasmas, great importance is attached to the GI test because of the high degree of specificity and workability of this method The rather low sensitivity which is an obvious disadvantage of the test, may to some extent be compensated for by using suboptimal growth conditions (7) or by increasing the amount of test serum. As observed in the present work, in the tests performed with *M cynos* (H831) these modifications may occasionally result in non-specific inhibition. Apart from this no cross reactions whatsoever between any of the strains compared in this study were found by the GI test.

The MI test is known to possess high

specificity as well as high sensitivity as borne out also by the results of the present study. The demonstration by the author that phosphatase activity can be utilized in this test further extends its potential field of application.

The indirect IMF test using unfixed colonies as antigen is likewise quite sensitive and generally very specific, no cross reactions between any of the strains examined being found in this study. From a technical point of view the IMF test has the additional advantage of being very simple and rapid to perform and accordingly it should have a high priority in studies of classification.

The double immunodiffusion test is well suited for the study of an antigenic relatedness between species. Preparation of the antigen solutions by the freezing and thawing technique gave from 1 to 4 precipitation lines which developed in the homologous test systems. In the case of *M. felminutum* (BEN) the failure to obtain any precipitation lines may be attributed to a low antigen concentration due to the fact that this strain can only be grown to a titre of about 10^6 c.f.u./ml.

On the basis of the results achieved by testing all strains possessing antigens in common against each other antigen formulae could be established (Table 5). In each formula, at least one antigen is present which is not represented in the formula of any of the other strains. It is remarkable that antigenic relatedness occurs within the group of glucose fermenting *Mycoplasma* strains and within the group of arginine metabolizing strains, but not between these two groups. This phenomenon has been observed earlier in the case of bovine mycoplasmas (6) and is essentially on line also with the studies reported by *Kenny* (8). On the other hand, it is noteworthy that an antigen is shared by the glucose positive strain HRC689 and *M. bovigenitalium* (PG11) which metabolizes neither glucose nor arginine.

The conclusion finally to be drawn on the basis of the present study, namely that none of the data presented will invalidate the classification of the already named species, was not unexpected and hence need no further comments. The confirmation of the distinctness of strain MH5408, as well as the contribution made to elucidate the taxonomic status of strain HRC689 however deserve a more detailed discussion.

Classification and species designation of MH5408 and related strains. Strain MH5408 was isolated by *Armstrong et al.* (3) from the throat of a dog and, together with two other canine isolates, one from the bladder (MH4603) and one of unknown source, it was found to belong to a separate group preliminarily referred to as serogroup D. Six strains isolated from the genital tract of male dogs were recently identified in this laboratory as serogroup D. According to a companion paper (12) strain MH5408 was found to belong to genus *Mycoplasma* and to metabolize arginine, but not glucose. In addition, the strain was phosphatase positive as well as film and spots positive. Since no cross reactions between MH5408 and other arginine positive *Mycoplasma* species were found by GI, MI and indirect IMF tests used in the present study, it is safe to conclude that serogroup D represents a distinct new species. It is proposed that this species be named *Mycoplasma opalescens* (L. part. adj. *opalescens* opalescent, referring to the opalescing film produced on solid medium). Strain MH5408, which has been deposited with the American Type Culture Collection (3) under the number 27921 and at the National Type Culture Collection under the number 10149 is herewith designated as the type strain of *M. opalescens*.

Taxonomic status of strain HRC689. This strain was isolated by *Barile et al.* (4) who suggested that it might represent a new species because it was distinct from other canine *Mycoplasma* species. The results of the present study show however that strain HRC689 is serologically related to *M. mycoides* subsp. *mycoides*, *M. mycoides* subsp. *capri*, Al Aubaidi & Fabricant Group L (1) and—to less extent—to Leach Group 7 (9). All of these organisms are mutually related and Groups L and 7 may represent separate

subspecies of *M mycoides* (6) Final classification of these groups, as well as of strain HRC689 must, however await further studies, including nucleic acid hybridization experiments. For the time being it is classified as *M mycoides*

REFERENCES

1 *Al-Aubaidi, J M & Fabricant J.:* Characterization and classification of bovine mycoplasma. Cornell. Vet. *61* 490–518, 1971
2. *Al Aubaidi J M Dardiri A H & Fabricant J* Biochemical characterization and antigenic relationship of *Mycoplasma mycoides* subsp. *mycoides* Freundt and *Mycoplasma mycoides* subsp. *capri* (Edward) Freundt. Int. J Syst. Bact. *22* 155–164 1972.
3. *Armstrong, D Tully J G., Yu B., Morton, V Friedman M H & Steger L.* Previously uncharacterized *Mycoplasma* isolates from an investigation of canine pneumonia. Infect. Immun. *1* 1–7 1970
4 *Barile M F Del Giudice R. A., Carski T R., Yamashiroya, H M & Verna J A.* Isolation and rapid identification of *Mycoplasma* species from canine tissues by plate immunofluorescence. Proc. Soc. exp. Biol. Med. *134* 146–148, 1970.
5 *Erno H & Stipkovits L.* Bovine mycoplasmas Cultural and biochemical studies. I Acta vet. scand. *14* 436–449 1973
6. *Erno H & Jurmanova, K* Bovine mycoplasmas Serological studies by double immunodiffusion, growth precipitation and growth inhibition. Acta vet. scand. *14* 524–537 1973
7 *Freundt E. A. Erno H Black F T Krogsgaard-Jensen A & Rosendal, S* Evaluation of reference reagents for mycoplasmas. Ann. N Y Acad. Sci. *225* 161–171 1973
8 *Kenny G E.* Serological comparison of ten glycolytic mycoplasma species. J Bact. *98* 1044–1055 1969
9 *Leach R. H.* Comparative studies of mycoplasmas of bovine origin. Ann. N Y Acad. Sci. *143* 305–316 1967
10. *Rosendal, S & Black F T.* Direct and indirect immunofluorescence of unfixed and fixed mycoplasma colonies. Acta path. microbiol scand Sect. B *80* 615–622, 1972
11 *Rosendal, S* Canine mycoplasmas II: Biochemical characterization and serological identification. Acta path. microbiol. scand. Sect. B, *82* 25–32 1974
12 *Rosendal, S* Canine mycoplasmas: Cultural and biochemical studies of type and reference strains. Acta path. microbiol. scand. Sect. B *83* 457–462 1975
13. Subcommittee on the Taxonomy of Mycoplasmatales Proposal for minimal standards for descriptions of new species of the order *Mycoplasmatales* Int. J Syst. Bact. *22* 184–188, 1972

Acta path. microbiol. scand. Sect. B, 83 471–476, 1975

IMMUNOCHEMISTRY OF A CELL WALL POLYSACCHARIDE ISOLATED FROM *EUBACTERIUM SABURREUM*, STRAIN L49

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Hofstad, T Immunochemistry of a cell wall polysaccharide isolated from *Eubacterium saburreum* strain L49 Acta path. microbiol. scand. Sect. B, 83 471–476, 1975

A polymer isolated from *E. saburreum* strain L49 contained O-acetylated glycero-galacto-heptose as the main constituent, and in addition an unknown aldose. The polymer was located at the outer part of the bacterial cell wall. The polymer reacted with antiserum to L49 microorganisms by precipitation and complement fixation. Two antigenic specificities were detected, one of which was destroyed by de-acetylation. The rabbit antibodies to both specificities were low-molecular-weight antibodies.

Key words *Eubacterium saburreum* cell wall polysaccharide immunochemistry

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A homoglycan, PS L44 composed of D-glycero-D-galacto-heptopyranose residues (3) was isolated from the anaerobic oral organism *Eubacterium saburreum*, strain L44 (6) The homoglycan, named PS L44 showed haptenic properties (7) Double diffusion in agar revealed two antigenic specificities, one of which was destroyed by de-acetylation. PS L44 was located at the surface of the bacterial cell (9) The antigen was present in eight of nine samples of dental plaque (8)

A novel heptose-containing cell wall polymer has been isolated from another strain of *E. saburreum*. The present report is concerned with the immunochemistry of this polymer

MATERIALS AND METHODS

Cultural Conditions

The filamentous organism *E. saburreum* strain L49 (4) and the other strains examined were grown in 1000 ml screw-cap bottles filled to the top with the following medium whale meat extract (Rieber & Søn A/S Bergen, Norway) 10 g, Proteose Peptone (Oxoid) 15 g, NaCl 5 g, KH_2PO_4 1.5 g, $Na_2HPO_4 \cdot 2H_2O$ 3.5 g $(NH_4)_2SO_4$ 0.5 g, Yeast Extract (Oxoid) 3 g, L-cysteine HCl 1 g, glucose 5 g, and distilled water 1 litre, pH 7.0, supplemented with 5 per cent horse serum. Following incubation at 37 C for two days the microbial cells were centrifuged, washed twice in saline and kept frozen until used.

Extraction and Purification Methods

Packed microbial cells or acetone dried cells were digested with trypsin (Trypsin 1 250 Difco) in 0.02 N tris-HCl buffer containing 0.05 N $CaCl_2$, pH 7.8. The digestions were carried out at 37 C for 20 h in a shaker and with an enzyme to substrate ratio of approx. 1:50 (w/w)

Sephadex G-75 (Pharmacia AB Uppsala, Sweden) was used for gel filtration. The columns were stabilized and eluted with 0.02 M phosphate buffer pH 7.4 containing 0.001 M EDTA and 0.02 per cent sodium azide. Ion exchange chromatography was carried out on columns of DEAE-cellulose (DEAE-SS Serva, Heidelberg, West-Germany) 0.02 M phosphate buffer pH 7.4 was used for elution. Column chromatography was also performed on hydroxyl apatite (Bio Gel HTP Bio Rad Laboratories, Richmond, California, USA). The hydroxyl apatite was equilibrated and eluted with 0.001 M phosphate buffer pH 6.8. Deacetylation of the purified polymer was obtained by treatment with 0.01 N NaOH for 60 min at 56 C.

Paper Chromatography

Acid hydrolysis was performed in sealed tubes with N H_2SO_4 for 4 h or with 3 N HCl for 3 h. The H_2SO hydrolysates were neutralized by passage through a column of Dowex 1 in the *formate* form, and acid was removed from HCl hydrolysates by evaporation *in vacuo* over NaOH pellets. Circular paper chromatography was run with a butanol-pyridine-water (6-4-3) (A) or ethyl acetate-acetic acid-water (3 1 1) (B). Sugars were stained with aniline hydrogen phthalate (13) or silver nitrate.

Chemical Analyses

The sulphuric acid-cysteine reaction according to *Dische* (1) was used for determination of heptose. D-glycero-D-galactoheptose served as standard. O-acetyl groups were determined by alkaline hydroxylamine (2) with acetylcholin-HCl as standard. Protein was measured by the Folin Ciocalteu phenol method (12). Bovine serum albumin was used as standard.

Analytical Ultracentrifugation and Immunoelectrophoresis

These analysis were performed as described previously (9).

Immunoferritin Technique

An indirect technique was used (8). In short undiluted antiserum-L49 which had been absorbed with a heterologous *E. saburreum* strain was added to a suspension of L49 microorganisms. Following incubation at room temperature (22 C) and repeated washings, a ferritin-IgG conjugate of goat anti-rabbit gammaglobulin (Cappel Laboratories Inc., Downington, Pa., USA) was added. Control experiments in which L49-antiserum absorbed with PS L49 or normal rabbit serum were substituted for untreated antiserum-L49 were included. The ferritin-labelled bacteria and the included control were fixed in 1 per cent osmium tetroxide in Kellenberger buffer and embedded in epoxy resin (Durcupan, Fluka AG Buchs SG Switzerland). Thin sections, collected on carbon-coated formvar membranes were stained with a saturated solution of uranyl acetate in 50 per cent (/\ ) ethanol in water and lead citrate (14).

Serological Methods.

Antisera were produced in rabbits by intravenous injections of whole microbial cells (5). Gel filtration of antiserum was carried out at room temperature (22 C) on a 86 cm high column of Sephadex G-200 internal diameter 2.5 cm, at an elution rate of 3 ml/cm²h. 0.1 M tris-HCl buffer pH 8.0 containing 1 M sodium chloride was used as eluant.

Double diffusion in agar gel and *ring test precipitation* were performed as described (6). Methods for *indirect haemagglutination* and *complement fixation* have been reported (5, 7). In complement fixation tests two 100 per cent lytic units of complement and two units of amboceptor were used. Tubes with 0.1 ml of serum dilution, 0.1 ml of antigen, and 0.2 ml complement were incubated overnight at 4 C, after which 0.2 ml of a 1 per cent suspension of sensitized sheep cells was added.

RESULTS

Preparation of the Polymer

Three different batches of L49 microbes were treated with trypsin 2 g acetone-dried cells (Batch 1) were digested with 40 mg trypsin in 400 ml buffer 4 g acetone-dried cells (Batch 2) were digested with 80 mg trypsin in 800 ml buffer and 20 g packed, wet cells (Batch 3) were digested with 80 mg trypsin in 400 ml buffer. The supernatant fluid following centrifugation gave 2 agar precipitation lines with antiserum L49 a heavy line produced by the cell wall polymer and a fainter line representing a contaminating precipitinogen.

Purification of the polymer named PS L49 was achieved employing the procedure used for preparation of the homoglycan PS L44 from tryptic digests of *E. saburreum* strain L44 (6). First the dialysed and concentrated supernatant fluid was passed through a 75 cm high and 4.5 cm wide column of Sephadex G-75 in order to separate PS L49 from low molecular-weight

substances. By ion exchange chromatography on DEAE-cellulose (column dimensions 1.8 × 44 cm (Batch 1 and 2) 2.8 × 55 cm (Batch 3)) which was the next step, PS L49 went straight through the column, while the contaminating antigen and varying amounts of ultraviolet light absorbing material were retained. In order to get rid of remaining amounts of protein PS L49 was finally passed through a 2 cm wide and 50 cm high column of Bio Gel HTP. Judged by titre, approximately 100 per cent of PS L49 was recovered from the extracts of Batch 1 and 2. Unknown amounts of PS L49 extracted from Batch 3 were accidentally lost during gel filtration.



Fig. 1 Immuno-electrophoresis (1 h, 250 V) of untreated (A) and de-acetylated (B) PS L49 1 mg/ml, against antiserum-L49 (C)

Criteria of Purity

By immunoelectrophoresis in agar gel untreated and NaOH treated PS L49 produced one precipitating arc against antiserum to L49 cells, situated at the cathodal side of the point of application (Fig. 1).

When examined by analytical ultracentrifugation, PS L49 sedimented as a single peak (Fig. 2). The uncorrected sedimentation coefficient (S_{20}) was 1.34.

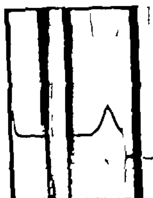


Fig. 2 Schlieren pattern of PS L49 5 mg/ml in 0.05 M phosphate buffer pH 6.8. Exposure at 72 min after reaching speed (59 780 rev/min, Spinco Model E, Rotor Ad-D)

Chemical Composition

Paper chromatography of PS L49 hydrolyzed with sulphuric acid revealed two distinct sugar components. The major sugar had the same mobility as D-glycero-D-galactoheptose in the solvent systems used. The other sugar moved like ribose (R_{gl} 1.4) in solvent system A, but gave the same red-brown colour with aniline hydrogen phthalate as the aldoheptose. In solvent system B, the mobility of the unknown was slightly less than that of ribose (R_{gl} 1.1). When hydrochloric acid was used for hydrolysis of PS L49 the band corresponding to the unknown sugar was weak.

The results of the quantitative analyses performed have been compiled in Table 1.

TABLE 1 *Quantitative Chemical Composition of the Cell Wall Polymer PS L49 from E. saburreum Strain L49*

Cells	Yield mg	Protein per cent	Heptose per cent	Heptose µmoles/mg*	O-acetyl µmoles/mg
Batch 1 acetone-dried, 2 g	25	4.8	62	3.0	2.5
Batch 2, acetone-dried, 4 g	20	2.9	52	2.5	1.5
Batch 3 packed, wet, 20 g	76	7.0	55	2.6	2.5

* Calculated.

Serological Properties

The serological activity of PS L49 against antiserum to whole L49 microorganisms is shown in Table 2. PS L49 did not sensitize sheep erythrocytes to agglutination in anti serum L49

TABLE 2. *Serological Reactivity of Untreated and De-acetylated (0.01 N NaOH 56° C 60 min) PS L49*

Treatment	Precipitating activity (ng/ml)*	Complement binding activity (ng)§
None	0.98	0.0156
De-acetylated	0.98	0.0156

* Lowest concentration of PS L49 (2 fold dilution) giving positive ring test.

§ Lowest amount of PS L49 (2-fold dilution) giving maximum serum titres.

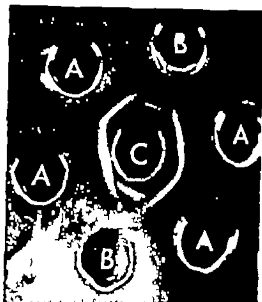


Fig 3 Agar precipitation bands produced by untreated (A) and de-acetylated (B) PS L49 0.5 mg/ml, against antiserum-L49 (C)

The agar precipitation band produced by untreated PS L49 against antiserum L49 spurred over that of the de-acetylated antigen (Fig 3) The precipitins reacting with de-acetylated PS L49 were removed from the antiserum by absorbtion with untreated PS L49 Following absorbtion with a surplus of de-acetylated PS L49 the antiserum still precipitated the untreated polymer Such an immunoprecipitate was washed and treated at 100 C for 2 min to denature the antibodies bound in it. Part of the heated precipitate was de-acetylated by treatment with sodium hydroxide. The heated and de acetylated immunoprecipitate produced one band by double diffusion in agar against antiserum L49 This band coalesced with that produced by de-acetylated PS L49 but was spurred over by the band given by the heated, but non-de-acetylated immunoprecipitate, or by untreated PS L49

Four ml of antiserum L49 was applied to the Sephadex G 200 column. The fractions were dialysed against buffer diluted 2 fold and examined for complement binding antibodies reacting with untreated or de-acetylated PS L49 (Fig 4) All antibodies to de-acetylated PS L49 and the majority of the complement-binding antibodies to untreated PS L49 were associated with the second protein peak. Fractions 26–28 (cf. Fig 4) produced a weak agar precipitation line against PS L49 0.01 mg/ml.

The specific PS L49 agar precipitation line

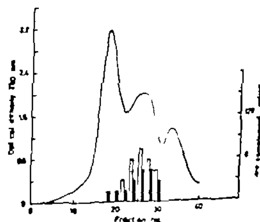


Fig 4 Fractionation of 4 ml antiserum-L49 on Sephadex G-200. Fractions 7 5 ml were collected Closed bars complement fixation titres against untreated PS L49 Open bars—complement fixation titres against de-acetylated PS L49

was not detected in extracts from 20 other oral strains of *E. saburreum*.

Location of the Polymer in the Microbial Cell

The majority of the L49 microorganisms treated with homologous antiserum or normal rabbit serum, and with the ferritin-IgG conjugat, were more or less disintegrated. Some of the cells which had maintained their original shape following incubation with antiserum-L49 absorbed with the heterologous *E. saburreum* strain and subsequent treatment with the conjugate were surrounded by masses of ferritin particles in close contact with the outermost cell wall layer (Fig. 5). Clumps of ferritin particles were also seen in contact with detached cell envelopes, or unconnected to visible cell material. A few clumps of ferritin particles were observed between the cells and unconnected to visible material in the preparation of L49 which had been incubated with homologous antiserum absorbed with PS L49.



Fig. 5. *Eubacterium saburreum* strain L49 treated with homologous rabbit antiserum and ferritin-labelled antibodies to rabbit gammaglobulin. Electron micrograph, × 60,000.

DISCUSSION

The heteropolymer PS L49 had much in common with the homoglycan PS L44 isolated from *E. saburreum* strain L44. Both polymers were extracted by treating whole microorganisms with trypsin, and behaved in the same way during the different steps of purification. Like PS L44, PS L49 is most likely located at the outer part of the bacterial cell (cf. Fig. 5) possibly in covalent linkage to protein.

O-acetylated galacto-glycero-heptose constituted approximately two-thirds of the PS L49 preparations. The molar ratio of O-acetyl to heptose varied from one preparation to another. Some O-acetyl may well have been lost during extraction and purification. The preparation isolated from L49 batch 5 showed a O-acetyl to heptose ratio of approximately 1. This suggests that, in the microbial cell, each heptose residue within the polymer carries one O-acetyl group. In addition to heptose the polymer contained an unknown aldoheptose. Preliminary structural studies have indicated that this sugar is a 6-deoxy-heptose (*Hoffman et al.*, unpublished). Thus PS L49 seems to have a more complicated chemical structure than the homoglycan PS L44.

PS L49 was highly active by precipitation and in complement fixation tests. Examination of the immunoprecipitate obtained by mixing untreated PS L49 with antiserum which had been absorbed with sodium hydroxide treated PS L49 showed that the two antigenic specificities revealed by double diffusion in agar were part of the same macromolecule. Also in PS L44, O-acetyl is part of one of two specificities. If present, O-acetylated sugar residues seem in fact to be determinants of the antigenic specificity of microbial cell wall polysaccharides (10, 11).

As determined by fractionation of antiserum L49 on Sephadex G-200 virtually all detectable antibodies present in the serum,

which had been drawn 4 weeks after immunization was started were low-molecular weight antibodies. The same was true of rabbit antiserum to the homoglycan PS L44

The absence of PS L49 in the 20 *E. saburreum* strains examined by double diffusion in agar against L49 antiserum indicates that PS L49 is an uncommon type antigen in oral *E. saburreum*

The electron microscopy was carried out by Dr. *Tryggve Lie* Research Laboratories, School of Dentistry University of Bergen.

REFERENCES

1. *Dische Z* Qualitative and quantitative colorimetric determination of heptoses. J biol. Chem. *204* 983–997 1953
2. *Hestrin S.:* The reaction of acetylcholine and other carboxylic acid derivatives with hydroxylamine, and its analytical application. J biol. Chem. *180* 249–261 1949
3. *Hoffman J., Lindberg B., Svensson S & Hofstad T* Structure of the polysaccharide antigen of *Eubacterium saburreum* strain L44 Carbohydr Res. *35* 49–53 1974
4. *Hofstad T* An anaerobic oral filamentous organism possibly related to *Leptotrichia buccalis* 1 Morphology some physiological and serological properties. Acta path. microbiol. scand. 69 543–548, 1967
5. *Hofstad T.:* Serological properties of lipopolysaccharide from oral strains of *Bacteroides melaninogenicus.* J Bact. *97* 1078–1082 1969
6. *Hofstad T* A polysaccharide antigen of an anaerobic oral filamentous microorganism (*Eubacterium saburreum*) containing heptose and o-acetyl as main constituents. 1 Isolation and chemical characterization of the antigen. Acta path. microbiol. scand. Sect. B, *79* 835–840 1971
7. *Hofstad T.:* A polysaccharide antigen of an anaerobic oral filamentous microorganism (*Eubacterium saburreum*) containing heptose and o-acetyl as main constituents. 3. Some serological properties. Acta path. microbiol. scand. Sect. B *80* 609–614 1972.
8. *Hofstad T.:* Presence in dental plaque of a polysaccharide antigen of the anaerobic oral filamentous organism *Eubacterium saburreum.* Scand. J dent. Res. *82* 82–84 1974.
9. *Hofstad T & Selvig, K. A.:* A polysaccharide antigen of an anaerobic oral filamentous microorganism (*Eubacterium saburreum*) containing heptose and o-acetyl as main constituents. 2. Physical properties and location in the bacterial cell Acta path. microbiol. scand. Sect. B *80* 129–134 1972.
10. *How M J., Brimacombe J S & Stacey M* The pneumococcal polysaccharides. In: Wolfrom, M. L. (Ed.) Advances in carbohydrate chemistry vol. 19 Academic Press, New York and London 1964 pp 303 358.
11. *Kotelko K Staub A M & Tinelli R.:* Étude immunochimique sur les Salmonella. VIII Role des groupements O acétyls dans la spécificité d facteur 0 5. Ann. Inst. Pasteur *100* 618–637 1961
12. *Lowry O H Rosebr ugh N J Farr A L & Randall R. J* Protein measurement with the Folin phenol reagent J biol. Chem *193* 265–275 1951
13. *Partridge S M* Aniline hydrogen phthalate as a spray reagent for hromatography of sugars. N ture *164* 443 1949
14. *Reynolds E. S* The use of lead citrate at high pH as an electron-opaque stain in electron microscopy J Cell. Biol. *17* 208–212, 1963

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O-ANTIGENIC SPECIFICITY OF LIPOPOLYSACCHARIDES FROM *BACTEROIDES FRAGILIS* SS *FRAGILIS*

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Hofstad, T O-antigenic specificity of lipopolysaccharides from *Bacteroides fragilis* ss. *fragilis*. Acta path. microbiol. scand. Sect. B, 83 477 481 197

Lipopolysaccharides (LPS) isolated from three strains of *Bacteroides fragilis* ss. *fragilis* were examined by indirect haemagglutination and inhibition of haemagglutination in unabsorbed and absorbed rabbit antisera. Six different antigenic specificities were detected. All serological activity was destroyed by oxidation of LPS with periodate.

Key words *Bacteroides fragilis* ss. *fragilis* O-antigenic specificity lipopolysaccharides.

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Lipopolysaccharides (LPS) isolated from *Bacteroidaceae* are macromolecular complexes of carbohydrate lipid and small quantities of polypeptide, which in the electron microscope appear as structured particles (5) *Fusobacterium* LPS (7) and possibly also LPS from *Leptotrichia buccalis* (1) exhibit O-antigenic specificity Sheep red cells sensitized with LPS purified from phenol/water extracts of *B fragilis* NCTC 9343 are agglutinated by homologous antiserum and normal human serum (4) The NCTC 9343 LPS and LPS isolated from two other strains of *B. fragilis* have been examined by haemagglutination and inhibition of haemagglutination in unabsorbed and absorbed rabbit antisera. The results presented here show that O-antigenic specificity is displayed also by *B. fragilis* LPS.

MATERIALS AND METHODS

Organisms

B. fragilis ss. *fragilis* strains NCTC 9343 Lille E 323 and SBL B55 were obtained from National Collection of Type Cultures, Central Public Health Laboratory London Institut Pasteur de Lille and National Bacteriological Laboratory Stockholm, respectively

Cultural Conditions

Cultures of E 323 and SBL B55 were grown in 500 or 1000 ml screw-cap bottles filled to the top with the following medium (g/l) Tryptone (Oxoid) 15 proteose peptone (Oxoid) 10 NaCl, 5 KH_2PO 1.5 Na_2HPO $2H_2O$ 3.5 $(NH_4)_2SO_4$, 0.5 yeast extract (Oxoid) 3 L-cysteine HCl, 1 and glucose, 5 pH 7.0 NCTC 9343 was grown in a chemostat at pH 7.0 and at dilution rates ranging from 0.05 to 0 11 h^{-1} (2) The medium was the same except that yeast extract was omitted and whale meat extract was substituted for tryptone.

Isolation of LPS

Washed organisms were suspended in distilled water to give 100 mg per ml, and extracted by

constant stirring with equal volumes of 90 per cent phenol for 15 min at room temperature (approx. 22 C). LPS was purified from the water phase by ultracentrifugation (100,000 × g for 60 min) and treatment with ribonuclease and deoxyribonuclease (6). Treatment of LPS with periodate was performed as described (5).

Gas Liquid Chromatography (GLC)

For detection and quantitative determination of aldoses, samples were hydrolysed in 0 1 M HCl at 100° C for 48 h. The hydrolysed samples were neutralized with Amberlite IRA 410 HCO form and the aldoses converted to alditol acetates by the method of *Sawardeker et al.* (8). GLC was run in a Perkin-Elmer 900 Gas Chromatograph with a flame ionization detector and fitted with a glass column (0.20 × 180 cm) packed with 3 per cent ECNSS-M (w/w) on Gas-Chrom Q,100–120 mesh (Applied Science Laboratories, State College Pennsylvania). The flow of gas (N_2) was 30 ml/h, the column temperature was 190 C and the detector temperature 260 C.

Paper Chromatography

was used for the demonstration of amino sugars (6).

Serological Methods

Antisera were produced in rabbits by intravenous injection of whole organisms (3). Before use the antisera were absorbed with sheep red cells. Sheep red cells were sensitized with LPS which had been treated with 0.25 M NaOH for 1 h at 56 C and neutralized with 0.25 M HCl. A 1 per cent suspension of sheep cells in phosphate buffered saline pH 7.2 (PBS) was sensitized (1 h at 37 C) with an equal volume of LPS suspension containing four times the least amount of antigen sensitizing erythrocytes for maximal agglutination in homologous antiserum.

Indirect haemagglutination (HA) was carried out in U-well plastic trays (Microtitre equipment, Flow Laboratories, Irvine, Scotland). To 0.025 ml volumes of antiserum diluted twofold was added 0.025 ml of a 0.5 per cent suspension of sensitized sheep cells. The PBS used as diluent contained 0.5 per cent bovine serum albumin as a stabilizer. Patterns of agglutination were read after refrigeration overnight. For inhibition of HA with LPS, 0.025 ml of serum dilution containing 8 agglutinating units of antiserum were pre-incubated at room temperature for 1 h with inhibitor in 0.025 ml of PBS. Absorption of antiserum was carried out by mixing 0.1 ml of serum with 0.1–0.6 mg of LPS in 0.9 ml of PBS. After refrigeration overnight, the absorbed samples of serum were cleared by centrifugation.

EXPERIMENTS AND RESULTS

The preparations of LPS from the 3 *B. fragilis ss. fragilis* strains contained the same neutral sugars but in different relative amounts (Table 1). Glucosamine and galactosamine were present in all LPS. The antibody neutralizing capacity and the red cell sensitizing ability of the preparations did not parallel each other.

Cross-reacting antibodies were found in antisera to all three strains (Table 2). The agglutination in the antisera of sheep cells sensitized with homologous LPS was not inhibited by any of the heterologous LPS. This indication of serological multispecificity harboured by each LPS was confirmed by absorption experiments (Table 3). Absorption of heterologous antisera removed the antibodies cross-reacting with the LPS used for absorption, but left antibodies of other specificities.

The results of the absorptions can be ex

TABLE 1 *Serological Reactivity and Sugar Composition of LPS Isolated from B. fragilis ss. fragilis Strains NCTC 9343, Lille E 323 and SBL B55*

LPS	Sensitizing activity µg/ml	Min. dose inhibiting HA§ µg	Neutral sugars, mole per cent Rha	Fuc	Glc	Gal	Man
NCTC 9343	3 12	2.5	1 7	4.6	87 3	5.5	0.8
Lille E 323	6.25	0 62	8.9	25 0	25 0	32.2	8 9
SBL B55	3 12	0 31	5.3	10 5	55.2	15.8	13 2

Least concentration of LPS to give maximal serum titres in homologous antiserum

§ 8 agglutinating units of homologous antiserum.

TABLE 2. *Titres in Indirect HA Tests of Antibody to LPS in Antisera to* Bacteroides fragilis ss. fragilis *Strains NCTC 9343 Lille E 323 and SBL B55*

LPS used for sensitization	Anti-9343	Anti-E 323	Anti-B55
LPS-9343	640	640	1280
LPS-E 323	160	640	320
LPS-B55	160	160	2560

plained if it is assumed that at least 6 different antigenic specificities or factors are present in the LPS preparations examined which henceforth are termed factor 2–7

Factor 2 Absorption of anti-9343 with LPS E 323 and of anti-E 323 with LPS-9343 eliminated the cross-reacting antibodies to LPS-E 323 and LPS 9343 respectively but both absorbed antisera still agglutinated sheep cells sensitized with LPS-B55 (Table, 3 rows (3) and (7)) Consequently LPS isolated from strains NCTC 9343 and Lille E 323 have an antigenic specificity (or more specificities) in common.

Factor 3 Absorption of anti 9343 with LPS-B55 and of anti-B55 with LPS 9343 removed cross-reacting antibodies to LPS-B55 and LPS-9343 respectively The absorbed antisera contained both antibodies which agglutinated sheep cells sensitized with LPS-E 323 (Table 3 rows (4) and (11)) In effect, NCTC 9343 LPS shares an antigenic specificity (or more specificities) with LPS isolated from strain SBL B55

Factor 4 Similarly anti-E 323 and anti-B55 which had been absorbed with LPS-B55 and LPS-E 323 respectively contained antibodies that reacted with sheep cells sensitized with LPS-9343 (Table 3 rows (9) and (12)) Therefore LPS from strain SBL B55 has an antigenic specificity (or more specificities) also in common with Lille E 323 LPS.

Factors 5 6 and 7 Specimens of antisera to strains 9343 E 323 and B55 absorbed with

TABLE 3. *Effect of Absorption with Various LPS on Titres in the Indirect HA Test of Antisera to* Bacteroides fragilis ss. fragilis *Strains NCTC 9343 Lille E 323 and SBL B55*

	Antiserum	Source of LPS used for absorption	Source of LPS used for sensitization: 9343	E 323	B55
(2)	Anti-9343	PBS control	320	320	160
(3)		9343	<20	<20	<20
(4)		E 323	160	<20	160
(5)		B55	160	40	<20
		E 323 + B55	160	<20	<20
(7)	Anti-E 323	PBS control	320	640	160
(8)		9343	<20	320	40
(9)		E 323	<20	<20	<20
(10)		B55	160	320	<20
		9343 + B55	<20	320	<20
(11)	Anti-B55	PBS control	1280	640	1280
(12)		9343	<20	640	1280
(13)		E 323	320	<20	640
(14)		B55	<20	<20	<20
		9343 + E 323	<20	<20	640

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TABLE 4 *Specificity of Test Systems for the Demonstration of the Antigenic Factors 2 7 of LPS Isolated from* B. fragilis *ss* fragilis *St mas NCTC 9343 Lille E 323 and SBL B55*

Factor	Test system Absorbed antiserum*	Sensitizing antigen§	Minimal inhibiting dose (µg) of LPS from 9343	E 323	B55
2	Anti-9343 abs. LPS-B55	LPS-E 323	2.5	2.5	>5
3	Anti-9343 abs. LPS-E 323	LPS-B55	2.5	>5	2.5
4	Anti-B55 abs. LPS-9343	LPS-E 323	>5	0.6..	0.62
5	Anti-9343 abs. LPS E 323 + B55	LPS-9343	2.5	>5	>5
6	Anti-E 323 abs. LPS9343 + B55	LPS-E 323	>5	1.25	>5
7	Anti-B55 abs. LPS-9343 + E 323	LPS-B55	>5	>5	0.62

* 8 agglutinating units of absorbed antiserum.
§ Sensitizing dose was 4 times the amount of LPS necessary to give maximal serum titre

LPS isolated from the two heterologous strains agglutinated sheep red cells sensitized with homologous LPS (Table 3 rows (5) (10) and (14)) This shows that, in addition to shared antigenic determinants, each LPS contained its own specificity Factor 5 is present in NCTC 9343 LPS factor 6 in LPS from Lille E 323 and factor 7 in LPS isolated from strain SBL B55

The absorption experiments gave some evidence of a presence of one or more determinants which were shared by all three LPS (factor 1) Thus, absorption of anti-9343 with LPS-B55 reduced its antibody titre against LPS-E 323 8-fold. Similarly absorption of anti-E 323 with LPS-9343 and of anti-B55 with LPS-E 323 caused a 4-fold reduction of titres in these antisera to sheep cells sensitized with LPS-B55 and LPS-9343 respectively (Table 3 rows (4) (7) and (12))

These interpretations give the following antigenic formulas for the three LPS preparations LPS-NCTC 9343 (1) 2, 3 5 LPS-Lille E 323 (1 2 4 6 LPS-SBL B55 (1) 3 4 7

In accordance with the results of the absorption experiments and their interpretations, test systems based on HA inhibition in absorbed antisera were constructed to be used for the demonstration of the antigenic factors 2–7 Table 4 shows the components of each system and the results of testing for specificity

Oxidation of the LPS preparations with periodate destroyed the inhibitory capacity All experiments and results were reproduced, using LPS isolated from different batches of bacteria and antisera raised in other rabbits.

DISCUSSION

The designation O antigen is used as a generic term for the antigenically active polysaccharide components of the LPS complex of Gram-negative bacteria The results of the present study show that LPS isolated from *B. fragilis* ss. *fragilis* have serological specificities analogous to O antigens from *Enterobacteriaceae* The sensitivity of the antigenic specificities to oxidation with periodate suggests that they are all harboured in the poly

ccharide moiety of the macromolecular LPS complex.

The LPS preparations from the three *B. fragilis* strains contained the same sugar components. The separate antigenic specificities may depend on variations in the structural arrangement or the sequence of the sugars within the polysaccharide moiety of LPS, or different sugars may be in end positions.

Possibly seven, at least six, distinct antigenic determinants or factors were disclosed, *i.e.* the maximal number to be detected in work with three strains and their antisera. One or more of these factors may be shared with LPS from other strains of *B. fragilis*. In addition, the LPS preparations examined may contain undetected specificities. Finally LPS from other *B. fragilis* strains may have factors not present in LPS isolated from the strains used in this study. Experiments using a larger number of strains are required in order to identify additional factors in LPS of *B. fragilis* and its subspecies.

REFERENCES

1. *Araujo W. C., de Varak E. & Mergenhagen S. E.* Immunochemical analysis of human oral strains of *Fusobacterium* and *Leptotrichia*. J Bact. *86* 837–844 1963
2. *Dalland Eli & Hofstad T.* Growth of *Bacteroides fragilis* in continuous culture and in batch cultures at controlled pH. Appl. Microbiol. *28* 856–860 1974
3. *Hofstad T.* Serological properties of lipopolysaccharide from oral strains of *Bacteroides melaninogenicus* J Bact. *97* 1078–1082, 1969
4. *Hofstad T.* Antibodies reacting with lipopolysaccharides from *Bacteroides melaninogenicus Bacteroides fragilis* and *Fusobacterium nucleatum* in serum from normal human subjects. J Infect. Dis. *129* 349–352, 1974
5. *Hofstad T.* Endotoxins of anaerobic Gram-negative microorganisms. In Balows, A., DeHaan, R. M., Dowell, V. R. & Guze, L. B. (Eds.) Anaerobic Bacteria Role in disease. Charles C. Thomas Publisher Springfield, Ill. 1974 p. 295–305.
6. *Hofstad T & Kristoffersen T.* Chemical characteristics of endotoxin from *Bacteroides fragilis* NCTC 9343 J gen. Microbiol. *61* 15–19 1970
7. *Kristoffersen T., Maeland J. A. & Hofstad, T.* Serologic properties of lipopolysaccharide endotoxins from oral fusobacteria. Scand. J dent. Res. *79* 105–112 1971
8. *Sawardeker J. S., Sloneker J. H. & Jeanes A.* Quantitative determination of monosaccharides as their alditol acetates by gas liquid chromatography Analyt. Chem. 37 1602–1604 1965

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QUANTITATION OF THE INHIBITORY EFFECT OF ERIOCHROME BLACK AND SODIUM NITRITE ON NON-SPECIFIC IMMUNOFLUORESCENT STAINING

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Karlsson, K.-A., Nilsson, G., Thore, A. & Morein, B. Quantitation of the inhibitory effect of eriochrome black and sodium nitrite on non-specific immunofluorescent staining. Acta path. microbiol. scand. Sect. B 83 482–490 1975

To evaluate non-specific staining (NSS) of animal tissues by FITC-labelled immunoglobulins a model system was developed. HeLa cells were treated with labelled antisalmonella globulins and the fluorescence intensity of the cells was determined quantitatively by means of a fibre optic probe system. This system was used to determine the optimal treatment conditions (adequate concentrations, duration of treatment) using the two NSS-reducing agents eriochrome black and sodium nitrite. Approximatively the same inhibitory effects (40–50 per cent using nitrite about 85 per cent using eriochrome black) were obtained by conjugates of different F/P ratio values. The fibre optic probe system was also used to determine the effects of the above-mentioned agents on the NSS of liver sections. In this system, the NSS was strongly reduced by eriochrome black whereas nitrite treatment did not induce any inhibitory effect. The applicability of nitrite and eriochrome black as NSS-reducing agents was further demonstrated by the fact that they had no influence on the specific fluorescence intensity of salmonella bacteria. The effect of eriochrome black was also studied in clinical specimens infected with salmonella or tularemia bacteria.

Key words Eriochrome black sodium nitrit Inhibitory effect Immunofluorescent staining

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The non-specific fixation of conjugates to tissue cells and debris often causes problems in the interpretation of immunofluorescence patterns. Measurements of the fluorescence emission from monolayers of cell cultures have revealed that the non-specific staining (NSS) is proportional to the fluorescein content of the conjugate (10). In bacteriological diagnostic work there are occasions where high concentrations of fluorescein in the working-dilution of the conjugates must be

ed. Such is the case, for instance, in preparations of conjugates which must cover a multitude of antigens within the salmonella group. Such conjugates will therefore give very high degree of NSS

Several procedures have been used to reduce the non-specific staining In the case of conjugates of high specific titres, dilution is the simplest and most effective way by which to reduce or eliminate NSS (10). Other well-known procedures by which to reduce NSS is to remove overlabelled conjugate molecules by absorption with tissue powder or fractionation by ion exchange chromatography (7). Several counterstaining techniques have been developed, for instance treatment with lissamine rhodamine RB-200 labelled serum proteins, Evan's blue, congo red or flazo orange. However some authors have noticed that the use of counter stains may reduce the specific immunofluorescence (3).

Recently *Burtin* & *Sabine* (1) reported that treatment of tissue specimens with sodium nitrite (1 per cent solution in 2 per cent acetic acid) before immunofluorescent staining (IF) markedly reduced the NSS. *Fey* (3, 4) has found that treatment with eriochrome black after the staining procedure is a simple method by which to reduce NSS in tissues infected with different bacterial or viral agents. These two methods are simple to perform and seem to have only a slight influence on the specific staining. The object of the present study was to evaluate in an accurate way the effect of these reagents using quantitative microfluorometry.

A model system consisting of HeLa cells stained with FITC-labelled antisalmonella globulins and treated with sodium nitrite and eriochrome black was developed. The influence of the agents on the specific immunofluorescent staining of salmonella bacteria was also determined.

MATERIAL AND METHODS

Cell cultures. HeLa cells were cultivated on cover slips in plastic Petri dishes to a density not exceeding 1 × 10⁴ cells/cm². The cells were incubated in Leibowitz medium complemented with calf serum, 10 per cent, penicillin, 100 IU/ml and streptomycin, 100 µg/ml. After a cultivation period of 1–2 days the cells were washed twice in PBS and then fixed in acetone for 30 minutes at room temperature. Residual acetone was removed by incubation for 45 minutes at 37 C.

Liver-tissue sections. Cryostat sections (5 µ) of mouse liver were fixed in acetone for 30 minutes at room temperature and residual acetone removed by incubation for 45 minutes at 37 C.

Bacterial antigens. Salmonella typhi murium (strain no. 3476, the National Veterinary Institute, Stockholm, Sweden) was used for the preparations. Antigens for the preparation of antiserum and for agglutination test were prepared as described earlier (12). Antigens for the immunofluorescent examination of smears were prepared from broth cultures grown for 24 hours at 37 C. The bacteria were collected by centrifugation, washed once and resuspended in saline to give a slight turbidity.

Antiserum against S. typhi murium was produced in rabbits according to methods described earlier (11).

Antibacterial conjugates of different fluorescein concentrations but with the same protein content (F/P ratio) were prepared from the γ-globulin fraction of the antiserum according to methods described earlier (12). The amount of fluorescein was determined according to the method of *McKinney et al.* (13) and the protein content according to the biuret method (8). The F/P ratios are expressed in µg fluorescein per mg of protein. The titres of the O- and H-agglutinins of the conjugates used were 1:160 and 1:2560, respectively. Unless otherwise stated, the conjugates were used at the working dilution of 1:10.

FITC-conjugated anti-rabbit globulin (National Bacteriological Laboratory, Stockholm, Sweden) was used in the indirect IF method and the F/P ratio of the conjugate was increased by further conjugation with FITC.

Specific staining of salmonella bacteria. Smears from the suspensions of the bacteria were fixed, stained by the direct IF method, washed, and mounted as described earlier (12).

Non-specific staining of HeLa cells and liver sections. Acetone-fixed preparations were stained, washed and mounted in the same way as for specific staining of salmonella bacteria.

Treatment of specimens with background reducing agents. Fixed HeLa cells, liver sections and salmonella bacteria were treated with sodium nitrite in 2 per cent acetic acid as described by *Burtin* & *Sabine* (1). The preparations were then washed twice in PBS and stained as described for specific staining of salmonella bacteria. Treatments with eriochrome black T (SIGMA) dissolved in distilled water were performed after the staining procedures (3). Treatments with flazo

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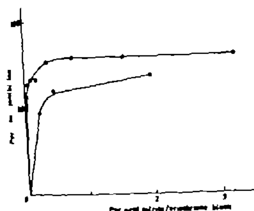


Fig. 2 Dose-response relationship for inhibition of NSS by nitrite and eriochrome black. Acetone-fixed HeLa cells were treated for 1 minute with different amounts of nitrite before the staining procedure or with eriochrome black for 15 seconds after the staining procedure. ○ = cells treated with eriochrome black. ● = cells treated with nitrite.

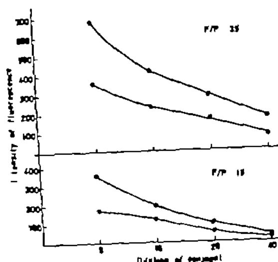


Fig. 4a. The nitrite-induced reduction of NSS at different dilutions and F/P values of the conjugate. The cells were treated with 1 per cent nitrite for 1 minute before the staining procedure ○ = untreated cells. ● = cells treated with nitrite.

microscope and connected to a photomultiplier and a chart recorder (Fig. 1) In some cells, the nucleus stained stronger and in others weaker than the cytoplasm. The NSS of the cells was reduced by treatment with nitrite or eriochrome black. All the treated cells showed a weaker staining of the nucleus than of the cytoplasm (Fig. 1)

The dose-response relationship with a view to a lowering of NSS by these agents revealed that concentrations of 0.5 per cent nitrite or 1 per cent eriochrome black are sufficient to produce a good reducing effect (Fig. 2)

The kinetics of inhibition is shown in Fig.

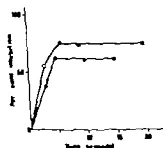


Fig. 3 Kinetics of inhibition. The cells were treated with 1 per cent nitrite before the staining procedure or with 3 per cent eriochrome black after the staining procedure. The treatment was terminated by rapidly pipetting a large volume of PBS over the slides. ○ = cells treated with eriochrome black. ● = cells treated with nitrite.

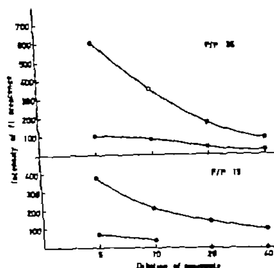


Fig. 4b The eriochrome-black induced reduction of NSS at different dilutions and F/P values of the conjugate. The cells were treated with 3 per cent eriochrome black for 15 seconds after the staining procedure ○ = untreated cells. ● = cells treated with eriochrome black.

TABLE 1 *The Effect of Eriochrome Black on the NSS after Indirect Staining*

Dilution of the conjugate	Per cent Inhibition of NSS
5	77
10	78
20	73

HeLa cells were treated with antisalmonella serum (diluted 1/10) followed by staining with anti-rabbit conjugate (F/P = 14). They were then treated with 3 per cent eriochrome black. Each value represents the mean of 20–30 cell measurements. The *variation* (S.E.) amounted to ±2–7 per cent of the mean.

3. Maximum inhibitory effects were reached 5–6 seconds after the start of the treatments.

The effects of nitrite and eriochrome black on the NSS at different F/P values and the effect of dilutions of the conjugate are demonstrated in Fig. 4. The inhibitory effects were of about the same magnitude (40–50 per cent inhibition using nitrite; about 85 per cent inhibition using eriochrome black) throughout the dilutions and F/P ranges used.

The effect of eriochrome black on NSS was also tested after indirect staining of HeLa cells with FITC labelled anti-rabbit globulin (Table 1). This treatment inhibited background fluorescence by about 75 per cent, which is comparable with the effect on the direct staining (Fig. 4b). Similar results were obtained by nitrite treatment.

The effect of treatment with eriochrome black on the NSS of liver sections after staining with FITC-labelled antisalmonella globulin was determined by the scanning procedure (Fig. 5). As in the case of HeLa cells, eriochrome black reduced the background fluorescence (about 60 per cent inhibition throughout the dilutions of the conjugate). A study of the kinetics of inhibition disclosed that maximum inhibitory effect was reached

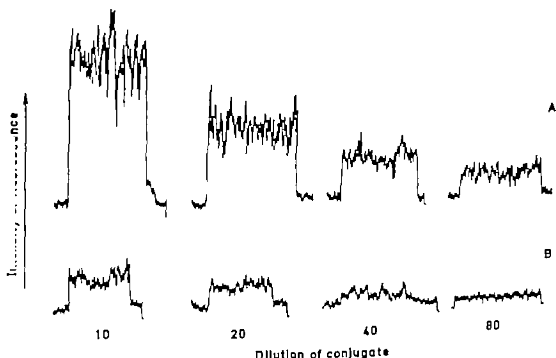


Fig. 5. Reduction of the NSS of liver slices using eriochrome black. Mouse-liver sections were fixed in acetone, stained with different dilutions of antisalmonella conjugate (*F/P* = 22) and treated with 3 per cent eriochrome black for 15 seconds. The NSS was determined by scanning over the slice using the fibre optic probe. A = untreated slices. B = eriochrome-black treated slices.

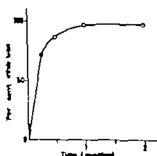


Fig. 6. Kinetics of inhibition. Mouse-liver sections were treated with 3 per cent eriochrome black after staining procedure. The treatment was terminated by rapidly pipetting a large volume of PBS over the sections.

ter 1 minute (Fig. 6) However using imprint smears, maximum effect was not reached until 20 minutes after application of eriochrome black (visual observation) A concentration of nitrite up to 5 per cent had no significant effect on the NSS of mouse-liver sections.

The applicability of nitrite and eriochrome black as background reducing agents was further tested by estimating their effect on the specific binding of antisalmonella conjugate to salmonella bacteria. The results of treatment of the bacteria with eriochrome black, nitrite, and the conventional counter stains flazo orange and Evan s blue are presented in Table 2. The specific binding of the conjugate was reduced by both flazo orange and Evan s blue. Nitrite or eriochrome black treatments did not significantly affect the specific binding

The usefulness of eriochrome-black treatments in practical applications of the IF technique could be demonstrated in clinical specimens (imprint smears made from internal organs and ulcerous fluid) infected with salmonella or tularemia bacteria (Fig 7)

The effect of nitrite and eriochrome black on the indirect specific staining of feline, panleukopenia virus infected lung cells with FITC-labelled anti-cat globulin was tested in preliminary experiments. In this system, nitrite and eriochrome black caused a certain inhibition of the specific staining (10 and 35 per cent, respectively)

DISCUSSION

The importance of using objective, quantitative methods to evaluate the effect of various techniques used in immunofluorescence is obvious. Until recently however the lack of simple measuring techniques has limited the work with quantitative immunofluorescence to relatively few specialized laboratories. The fibre optic probe system described by *Taylor et al.* (17 18) and used in the present study offers an inexpensive and simple way of measuring immunofluorescence. The system was considered well-suited for an investigation of non-specific fluorescence, since it affords the possibility to measure the fluorescence of individual cells, and even details of

TABLE 2. *The Effect of Different Treatments on the Specific Staining of* Salmonella typhi murium *with Antisalmonella Conjugate (F/P = 22)*

Treatment	Intensity of fluorescence Dilution 40	80	160
None	98 ± 3.1 (4+)	77 ± 7.0 (4+)	30 ± 2.3 (3+)
Eriochrome black	100 ± 4 1 (4+)	78 ± 3.7 (4+)	20 ± 1 7 (3+)
Nitrite	98 ± 9 1 (4+)	60 ± 4.0 (4+)	29 ± 2.1 (3+)
Flazo orange	43 ± 3 9 (3+)	19 ± 1.6 (2+)	4 ± 0.3 (1+)
Evan s blue	47 ± 3.5 (3+)	32 ± 2.3 (3+)	16 ± 1 4 (2+)

Values within parentheses = visual estimation of fluorescence intensity Values outside parentheses = instrumental estimation of fluorescence intensity (Mean ± S.E. of 10–20 randomly selected bacteria)

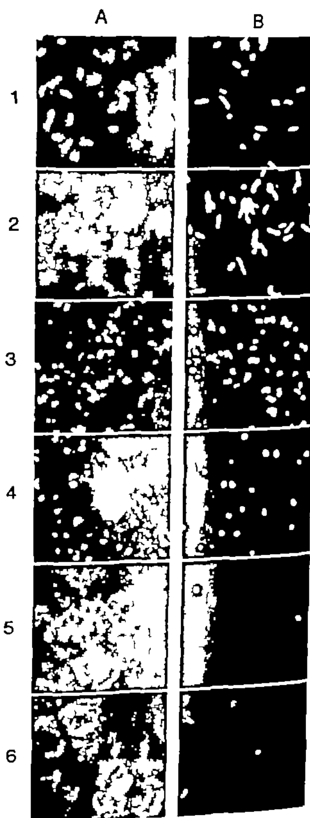


Fig 7 Reduction of the NSS of clinical specimens with eriochrome black. Series A comprises imprint smears stained with FITC labelled antisalmonella or anti *F tularensis* globulins. Series B comprises duplicate smears stained as above and posttreated with 5 per cent eriochrome black for 20 minutes. 1: Liver (mouse) infected with *Salmonella typhi murium*. 2 Spleen (mouse) infected with *Salmonella typhi murium* 3: Bone marrow (hare) infected with *Francisella tularensis*. 4: Liver (hare) infected with *Francisella tularensis*. 5 Lymph node (man) infected with *Francisella tularensis*. 6: Ulcerous fluid (man) containing *Francisella tularensis*. Magn. × 850.

)

ther. The method eliminates the necessity of preparing homogenous cell smears or counting the number of cells which previously was necessary in this kind of study since measurements were made on entire fields of vision in the microscope (5 16) In addition, the described technique allows a quantitation of differential staining of the cells which is demonstrated by the scannings shown in Fig. 1 where cytoplasmic and nuclear fluorescence are readily distinguished.

The results of this study clearly demonstrate and confirm the earlier finding that eriochrome black is a promising agent for the reduction of non-specific fluorescence in bacterial systems and probably also in viral systems. Nitrite treatments, on the other hand, seems to be most useful for the reduction of SS by immunofluorescent staining of viral antigens in tissue cultures. The agent may however not be suitable for demonstration of bacteria in infected tissue specimens, since it could not reduce the NSS of liver tissue sections.

The results also demonstrate that, when using NSS-reducing agents, it is important to evaluate treatment conditions such as adequate concentrations of the agents and the duration of the treatment.

Little is known of the mechanism of action of the NSS-reducing agents eriochrome black and nitrite. Nitrite has been proposed to reduce the unspecific affinity of FITC conjugates to positively charged cell constituents, principally basic amino groups in proteins (1, 14 15) Since the Gram-negative bacterial cell wall has a non-protein character nitrite would not be expected to affect bacterial immunofluorescence. This seems to be verified in the experiment shown in Table 2.

The inhibitory effect of eriochrome black has been proposed to be due to an absorption of emitted fluorescence (3 4) The reason why such an effect should be observed in the case of NSS but not in the case of bacterial specific immunofluorescence might possibly be a difference in affinity of the dye to cellular and Gram-negative bacterial surfaces. This difference possibly reflects the binding of the azocolour to the protein components of the animal cell membrane. In this context, the relatively great inhibitory effect of eriochrome black on the specific viral immunofluorescence might indicate that the viral antigens more closely correspond to cellular membrane components.

REFERENCES

1 *Burtin P & Sabine M.-C.*, Inhibition of the non-specific fixation of fluorescent globulins. Rev Europ. Études Clin. et Biol. *17*: 76–77 1972.

2. *Caspersson T Zech, L. Modest E. J Foley G E., Wagh U & Simonsson, E.*: Chemical differentiation with fluorescent alkylating agents in *Vicia Faba* metaphase chromosomes. Exptl. Cell. Res. *58* 128–140 1969

3 *Fey H*: Eriochrome Black, a means for reduction of non-specificity in immunofluorescence. Path. Microbiol. 38 271–277 1972.

4 *Fey H.*, Eriochromschwarz, ein Mittel zur Reduktion der Unspezifität bei der Immunofluoreszenz. Path. Microbiol. *39* 22–23 1973

5 *Ferreira U.*, Characterization of FITC-labelled $F(ab')_2$ fragments of IgG and a rapid technique for the separation of optimally labelled fragments. J Immunol Meth. *2*: 183–195, 1972.

6 *Goldman, M* Fluorescent Antibody Methods. Academic Press, New York and London, 1968, pp. 115–117

7 *Goldstein G., Slizys I & Chase M W* Studies on fluorescent antibody staining. I. Non-specific fluorescence with fluorescein-coupled sheep anti-rabbit globulins. J Exp. Med. *114* 89–109 1961

8. *Gornall, A G., Bardawill D J & David M M* Determination of serum proteins by means of the biuret reaction. J Biol. Chem. *177* 751–766, 1949

9 *Hall, T C & Hansen P A.*: Chelated azo dyes used as counterstains in the fluorescent antibody technic. Zbl. Bakt., I. Abt. Orig. *184* 548–554 1962

10. *Hebert G A., Pittman B. & Cherry W B.* Factors affecting the degree of nonspecific staining given by fluorescein isothiocyanate—labelled globulins. J Immunol. 98 1204–1212, 1967

11 *Karlsson K A & Thal, E.* Evaluation of the immunofluorescent reagents in the identification of salmonella bacteria. Zbl. Bakt. Hyg. I Abt. Orig. *A 228*: 474–481 1974.

12. *Karlsson K.-A. Thore A & Kudynowski J.* Quantitative studies of direct ...

immunofluorescent staining of salmonella bacteria. Acta Path. Microbiol. Scand. Sect. B. 83 17–24 1975
13 *McKinney R. M., Spillane J T & Pearce G H* Fluorescein diacetate as a reference color standard in fluorescent antibody studies. Anal. Biochem. 9: 474–476, 1964
14 *Mayersbach H & Schubert G.:* Die unspezifischen Reaktionen zwischen markierten Seren und Geweben bei der immunohistologischen Technik. Acta histochem. 10 44–82, 1960
15 *Nerin R. C., Richmond H G & Fothergill, J E.,* Differences in staining of normal and malignant cells by non-immune fluorescent protein conjugates. Brit. Med. J 2 1341 1343 1960.
16. *Pittman B., Hebert G A Cherry W B & Taylor G C.,* The quantitation of nonspecific staining as a guide for improvement of fluorescent antibody conjugates. J Immunol. 98 1196–1203 1967
17 *Taylor C E. D., Heimer G. I & Lidwell, O M.,* Use of a fibre optic probe for quantitative immunofluorescence studies. Lancet, 1 785–786 1971
18. *Taylor C E. D & Heimer G. I.,* Measuring immunofluorescence emission in terms of standard international physical units. J Biol. Standardization 2 11–20 1974

Acta path. microbiol. scand. Sect. B, 83 491–505 1975

THE ULTRASTRUCTURAL DEVELOPMENT OF THE MACROGAMETE AND FORMATION OF THE OOCYST WALL OF *TOXOPLASMA GONDII*

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Ferguson, D J P Hutchison, W M & Siim, J Chr The ultrastructural development of the macrogamete and formation of the oocyst wall of *Toxoplasma gondii*. Acta path. microbiol. scand. Sect. B, 83 491 505 1975

The macrogametes of *Toxoplasma gondii* develop within the epithelial cells of the cat ileum. As they develop the nucleus enlarges and dense patches of chromatin which were present in the nucleoplasm, disappear. Polysaccharide granules and lipid globules appear in the cytoplasm and increase in number during development. The wall-forming bodies of Type I (WFB I) appear before the wall-forming bodies of Type II (WFB II) WFB I are smaller more osmiophilic and more numerous than the WFB II The WFB I appear to form from vesicles produced by the smooth endoplasmic reticulum, and the WFB II form within the lacunae of the rough endoplasmic reticulum. Double membraned vacuoles appear to form from the nuclear membranes but the function of these is unknown. Throughout development the macrogamete retains a normal pellicle which possesses numerous micropores The first evidence of oocyst wall formation is the appearance of participate matter in the parasitophorous vacuole which precipitates to form Layer 1 of the oocyst wall. Layers 2 and 3 are unit membranes which form between Layer 1 and the pellicle During this development the organism has an organelle complement similar to that of the macrogamete Layers 4 and 5 form between Layer 3 and the pellicle. Layer 4 is osmiophilic and its formation is accompanied by the disappearance of WFB I Layer 5 is less osmiophilic than Layer 4 and its formation is accompanied by the disappearance of WFB II The two innermost layers (Layers 4 & 5) in the oocyst wall of *Toxoplasma* are similar to those found in *Isospora spp* and *Eimeria spp* Toxoplasma seems to be unusual in that, firstly it possesses an additional 3 layers and, secondly all 5 layers are formed outside the pellicle of the original macrogamete.

Key words *Toxoplasma gondii* macrogamete development; oocyst wall formation; ultrastructure

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Prior to 1969 only the exo-enteric stages of *Toxoplasma gondii* were known. These two stages were found in many mammals and birds and are now known as endozoites and cystozoites (*Hoare* 1972) Both stages repro-

duce asexually by the process of endodyogeny.

The discovery of the endo-enteric development in the domestic cat indicated that *Toxoplasma* was a coccidian parasite with the ability to undergo both asexual and sexual development in the epithelial cells of the small intestine (*Hutchison et al.* 1969, 1970; *Siim et al.* 1969; *Sheffield & Melton* 1970; *Frenkel et al.* 1970; *Piekarski & Witte* 1970; *Overdulve* 1970).

The asexual multiplication and microgametogony taking place in the intestine of the cat have been described at the ultrastructural level by *Sheffield* (1970), *Colley & Zaman* (1970), *Piekarski et al.* (1971), *Pelster & Piekarski* (1971) and *Ferguson et al.* (1974).

The ultrastructure of the macrogamete has been studied by *Colley & Zaman* (1970) and *Pelster & Piekarski* (1972). However, the development of organelles peculiar to the macrogamete has not been described and it is our intention to do so in this paper. In addition, the development of the oocyst wall, and the cytoplasmic changes occurring in the organism as it develops into an oocyst within the epithelial cells of the cat's intestine will be described.

MATERIALS AND METHODS

Specific Pathogen Free (SPF) cats were obtained and treated in a manner similar to that described by *Hutchison et al.* (1971). All cats were examined serologically and were Dye Test negative prior to the experiment. They were fed aseptic mouse brains containing the cystozoites of either the Beverley or the SSI/119 Strain of *Toxoplasma gondii*. Seven days later they were autopsied; all had become Dye Test positive by this time. The small intestine was immediately removed and divided into portions. A part of each portion was prepared for histological examination, as described by *Hutchison et al.* (1971), to determine the areas suitable for an electron microscope study. The adjacent part of each portion was divided into 1 mm cubes and fixed in 5 per cent glutaraldehyde in cacodylate buffer pH 7.2, for a minimum of 4 hours at 4° C. The tissue was then washed in cacodylate buffer for 12 hours and postfixed for 2 hours at room temperature, in 1 per cent solution of osmium tetroxide dissolved in the cacodylate buffer. The tissue was then dehydrated through a series of ethanol and 2 changes of propylene oxide and embedded in Araldite. Thin sections were cut with the LKB microtome, stained in uranyl acetate and lead citrate and examined in an AEI EM6B electron microscope at 60 kv.

Abbreviations in Figures

C	– Conoid
CA	– Canaliculi
ER	– Rough Endoplasmic Reticulum
G	– Golgi Body
HM	– Host Cell Mitochondrion
HN	– Host Cell Nucleus
L	– Lipid
M	– Mitochondrion
MP	– Micropore
MV	– Microvilli of Host Cell
N	– Nucleus
NU	– Nucleolus
OW	– Oocyst Wall
PG	– Polysaccharide Granules
PL	– Layers of the Pellicle
PV	– Parasitophorous Vacuole
SER	– Smooth Endoplasmic Reticulum
V	– Double-Membraned Vacuoles
WFB I	– Wall-Forming Bodies of Type I
WFB II	– Wall-Forming Bodies of Type II
1	– Layer 1 of Oocyst Wall
2	– Layer 2 of Oocyst Wall
3	– Layer 3 of Oocyst Wall
4	– Layer 4 of Oocyst Wall
5	– Layer 5 of Oocyst Wall

RESULTS

The merozoite enters a host epithelial cell and develops into the trophozoite within a thick walled *parasitophorous* vacuole (Fig. 1). The organism undergoes dedifferentiation with the loss of the rhoptries and micronemes

Fig. 1. A trophozoite showing the conoid, micropore and the nucleus which contains a nucleolus and dense patches of chromatin. × 20,000.

Fig. 2. An early macrogametocyte showing the large nucleus and nucleolus, rough endoplasmic reticulum and elongate mitochondria round the periphery. Note the absence of dense patches of chromatin in the nucleoplasm. × 13,500.

Fig. 3. An early macrogametocyte showing the nucleus, nucleolus, Golgi body, rough and smooth endoplasmic reticulum, a few WFB I and a number of polysaccharide granules and lipid globules. × 16,500.



C
MV
C.T.
N
L
N
NU
MV
ER
NU
L

although the conoid appears to remain intact (Fig 1) and is still visible in sections through the mature macrogamete (Fig 11) In the trophozoites which develop into macrogametocytes, the nucleus and nucleolus enlarge the dense patches of chromatin disappear (cf Figs. 1 & 2) but no division takes place Organisms possessing such nuclei will become macrogametocytes their mitochondria at this early stage are elongate structures situated around the periphery (Fig 2)

As development proceeds, storage materials (lipid globules and polysaccharide granules) and a few wall-forming bodies of Type I (WFB I)* appear in the cytoplasm which also contains a Golgi body smooth and rough endoplasmic reticulum and a number of mitochondria (Fig 3) There is a progressive increase in the amount of storage materials and of WFB I as development of the macrogametocyte proceeds. Wall-forming bodies of Type II (WFB II) are next to appear and increase in size as the macrogametocyte develops into the macrogamete.

In the mature macrogamete are found large amounts of storage material, numbers of WFB I and a few large WFB II (Fig 4) The cytoplasm also contains canaliculi, which are membrane bound fissures, mitochondria, and rough and smooth endoplasmic reticulum. Throughout the development, the structure of the nucleus has been unchanged with a single large nucleolus and amorphous nucleoplasm. Close to the nucleus are found double membraned vacuoles (Figs. 4 & 12) The organism is enclosed by a normal two layered pellicle which possesses numerous micropores. Although not shown in the figures, up to three micropores were observed in a single 50 nm thick section.

The structure of the mature macrogamete is represented diagrammatically in Fig 14

Development of Cytoplasmic Organelles Peculiar to the Macrogamete

a) *Double membraned vacuoles* These vacuoles appear to be formed by budding off from the nucleus (Fig. 12) and are normally spherical although elongate forms were observed (Fig 10) Some contain material similar in density to the nucleoplasm, while others contain more osmiophilic material (Fig. 13)

b) *WFB I* The WFB I appear to develop in close association with the smooth endoplasmic reticulum. Small vesicles, which were observed budding off from the membranes of smooth endoplasmic reticulum (Fig. 5) enlarge and their contents become more osmiophilic to form the WFB I (Fig 7) The WFB I had an average size of 0.35 μm and were limited by a single unit membrane, which was not always visible due to the osmiophilic contents (Fig 9)

c) *WFB II* The WFB II start to form within the lacunae of the rough endoplasmic reticulum, usually at the extremity of a strand (Fig 6) In this situation they continue to grow reaching a size of 1.2 μm. These mature WFB II are less osmiophilic and less numerous than the WFB I they are limited by a membrane of rough endoplasmic reticulum, below which is a unit membrane (Fig 8)

Dense bodies were found on rare occasions

* We use the terminology proposed by *Scholtyseck et al.* (1969 b)

Fig 4 A mature macrogamete showing the nucleus, nucleolus, numerous lipid globules, and polysaccharide granules, a number of WFB I and WFB II canaliculi and mitochondria. Note the vacuoles of variable density close to the nucleus. × 15,000.

Fig 5 Section through macrogametocyte showing a vesicle being formed from the smooth endoplasmic reticulum (arrow) × 40,000.

Fig 6. Section through macrogametocyte showing a WFB II forming within the lacunae of the rough endoplasmic reticulum. × 40,000

Fig 7 Section showing the vesicles produced by the smooth endoplasmic reticulum (Fig. 5) enlarging and developing osmiophilic contents to form the WFB I × 40,000.

Fig 8 A section through a WFB II showing its amorphous contents enclosed by a unit membrane (arrow) and a membrane of rough endoplasmic reticulum × 65,000

Fig 9 A section through WFB I showing its osmiophilic contents enclosed by a unit membrane (arrow) × 120 000


WFB II
PG
MI
CA
V
NU
N
L
WFB I
ER
WFB I
ER
WFB I
ER
WFB I
ER
WFB II
WFB I

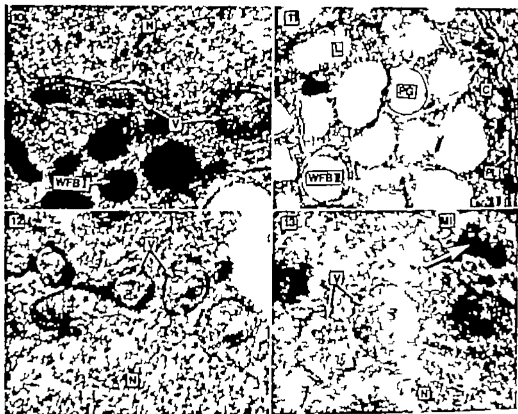


Fig 10 A section showing the variable shape of the double-membraned vacuoles close to the nucleus. × 36,000

Fig 11 A section through a mature macrogamete showing that the conoid is still present. × 25,000

Fig 12 A section showing the formation of the double membraned vacuoles from the nucleus. × 34,000.

Fig 13 A section showing the varying density of the contents of the double membraned vacuoles. Note the dense body within the mitochondrion (arrow) × 60,000

within the mitochondria of the macrogamete (Fig 13) Similar structures were occasionally found within the mitochondria of schizonts and microgametocytes.

Oocyst Wall Formation

The process of fertilisation of the macrogamete has not been observed. The first evidence of oocyst wall formation is the appearance of particulate matter in the parasitophorous vacuole (Fig 16) which precipitates to form a thick layer outside the pellicle of the macrogamete (Fig 17) This represents Layer 1 of the oocyst wall.

The ultrastructure of the organism, at this time is similar to that of the macrogamete (Fig 15)

After completion of Layer 1 a unit membrane forms below it (Fig 19) this represents Layer 2 of the oocyst wall. Layer 3 is another unit membrane which subsequently forms between Layer 2 and the pellicle, (Fig 20) Layers 2 and 3 can be seen to be unit membranes in Fig 23

During the development of these layers, membranes of rough endoplasmic reticulum were present just below the pellicle (Figs 17 & 19) No extensive cytoplasmic changes were observed in the structure of this developmental stage of the organism (Fig 18) both WFB I and WFB II were still present

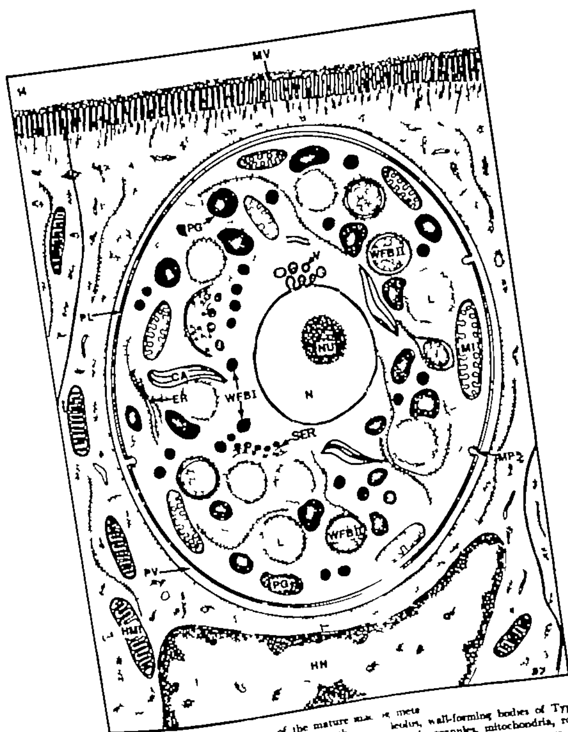


Fig 14 A diagrammatic represen n of the mature mac e mete
The mature macrogamete consi of nucleus w th leolus, wall-forming bodies of Type I and
Type II double-membraned ac les, lipid droplets, polysac haride granules, mitochondria, rough and
smooth endoplasmic reticulum nd canalicul enclosed w thin a pellicle which has more than one
m cropore

although the double membraned vacuoles were not observed.

The formation of Layer 4 is initiated by the appearance of a unit membrane between Layer 3 and the pellicle. The space between Layer 3 and this membrane is filled with granular material (Fig 22) This material coalesces to form Layer 4 which appears as an osmiophilic layer approximately 12 nm thick (Fig 23) When the organism was examined at this time it was found that the WFB I had disappeared, although the WFB II were present (Fig 21)

Layer 5 forms last of all below Layer 4 It is less osmiophilic than Layer 4 (Fig. 24) and is approximately 14 nm thick It appears to be limited on either side by a unit membrane (Fig 26)

At this stage the organism possesses neither WFB I nor WFB II but contains a nucleus with its nucleolus, mitochondria, and large numbers of polysaccharide granules and lipid globules (Fig 25)

From this it would appear that WFB I are concerned with the formation of Layer 4 and WFB II are concerned with the formation of Layer 5 Since these layers form outside the organism's pellicle the material from the WFB I and WFB II must pass through the pellicle before coalescing to form Layers 4 and 5 respectively

The development of the oocyst wall and associated cytoplasmic changes are summarized diagrammatically in Fig 28.

The later stages in oocyst development could not be examined because of changes in the nature of the oocyst wall. This prevented penetration and proper polymerisation of the embedding agent which was Araldite These changes were found to occur between Layers 4 and 5 of the oocyst wall (Fig 27)

DISCUSSION

Scholtyseck et al (1969 a) and *Scholtyseck et al.* (1971 a) working with *Eimeria tenella* and *E falciformis* respectively are of the opinion that an organism which is developing into a macrogamete can not be identified before the appearance of typical cytoplasmic organelles, i.e. the wall-forming bodies. However we now feel that the developing macrogamete of *Toxoplasma* can be identified at an earlier stage due to changes in the nuclear structure. Similar changes in the nuclear structure were used by *Speer et al.* (1973 b) to identify macrogametes of *Eimeria magna*.

The retention of the normal pellicle throughout the development is similar to that reported for *Toxoplasma* by *Pelster & Piekarski* (1972) and for *Isospora spp* by *Pelster* (1973) but differs from that of *Eimeria spp* where varying degrees of degeneration of the inner membranes may occur (*Scholtyseck et al.* 1971 b)

We could not confirm the findings of *Colley & Zaman* (1970) that the macrogametes of *Toxoplasma* possess intra vacuolar tubules these are thought to be a characteristic of the macrogametes of *Eimeria spp* (*Scholtyseck* 1973) *Pelster & Piekarski* (1972) and *Pelster* (1973) did not find the intra-vacuolar tubules in either *Toxoplasma or Isospora spp*.

Fig 15 An early oocyst showing the nucleus, polysaccharide granules, lipid globules, double-membraned vacuoles and WFB I and II all enclosed by a pellicle outside of which is a dense layer. × 10,000.

Fig 16 Section showing the particulate matter in the parasitophorous vacuole which is precipitating to form a layer outside the pellicle (arrows) × 51,000.

Fig 17 Part of an organism similar to that in Fig 15 showing the formation of Layer 1 of the oocyst wall outside the pellicle. *Note the membrane* of rough endoplasmic reticulum below the pellicle. × 90,000

Fig 18 An early oocyst showing the nucleus, polysaccharide granules, lipid globules and WFB I and II enclosed by a pellicle outside of which are three layers of the oocyst wall. × 12,500.

Fig 19 Part of an organism showing the formation of Layer 2 between Layer 1 and the pellicle. Note the membrane of rough endoplasmic reticulum below the pellicle. × 78,000

Fig 20 Part of an organism similar to that in Fig 18 showing the presence of Layers 1 2 and 3 outside the pellicle. × 150 000



WFB II
WFB I
N
PC
LI
CA
PL
PV
ER

The WFB I were found to be smaller than the WFB II which confirms the results of *Scholtyseck et al* (1971 b) and *Pelster & Piekarski* (1972) for *Toxoplasma* but the former reported that the WFB II were more numerous than the WFB I which is the opposite to that found by us. WFB I were found to appear before the WFB II but in *Eimeria spp.*, *Scholtyseck et al* (1971 b) found that WFB II were the first to form

The structure of the WFB I are denser than those identified by *Scholtyseck et al.* (1971 b) and *Pelster & Piekarski* (1972) for *Toxoplasma* but it is possible that this is due to difference in the preparation of the material. The WFB I of *Toxoplasma* are similar in size to those of *Eimeria falciformis* (*Scholtyseck et al* 1971 a) and *E acervulina* (*Lee & Millard* 1971) In these cases the WFB I are much smaller than the WFB II

The method of formation of the WFB I is unknown in *Eimeria spp* (*Scholtyseck* 1973) but in this study they appear to form from vesicles budded off from smooth endoplasmic reticulum. This has not previously been reported.

WFB II formation in *Eimeria spp* has been reported to occur within lacunae of the rough endoplasmic reticulum and Golgi body (*Scholtyseck* 1973) The former position appeared to be the site of formation in *Toxoplasma* and no evidence was found for the involvement of the Golgi body The WFB II were found to be much larger than those reported by *Scholtyseck et al.* (1971 b) for *Toxoplasma*. The structure of the WFB II with its amorphous contents, is similar to that reported by *Scholtyseck et al* (1971 b) *Colley & Zaman* (1970) and *Pelster & Piekarski* (1972) for *Toxoplasma* by *Pelster* (1973) for *Isospora spp* and by *Speer et al* (1973 b) and *Scholtyseck et al* (1971 a) for *E. magna* and *E. falciformis* respectively This differs from the structure of the WFB II of other *Eimeria spp* where the contents had a loose or mesh-like appearance (*Scholtyseck* 1962 & 1963 a *Colley* 1967 *McLaren* 1969 *Scholtyseck et al* 1966 *Scholtyseck et al.* 1969 a *Mehlhorn* 1972)

In certain *Eimeria spp* it has been proposed that dense bodies within the mitochondria may be involved in the formation of wall forming bodies (*Scholtyseck* 1973) Similar bodies were found in the mitochondria of the macrogamete of *Toxoplasma* but they were also found in the mitochondria of other developmental stages. It would thus appear that in *Toxoplasma* they may not be involved in wall-forming body formation.

The double-membraned vacuoles found in the macrogametes are formed from and enclosed by the two nuclear membranes. This differs from that found in the endozoite of *Toxoplasma* where vacuoles are formed from and enclosed by only the outer nuclear membrane (*Vivier & Petitprez* 1972) These double membraned vacuoles are similar to those described for the macrogametes of *Eimeria acervulina* and *Cryptosporidium* *Caryospora* *microcosm*

Fig 21 A developing oocyst which contains a nucleus, nucleolus, polysaccharide granules, lipid globules and WFB II Note the absence of WFB I. × 10,500.

Fig 22 Portion of the organism in Fig. 21 showing the unit membrane outside the pellicle (arrow) and the particulate matter between it and Layer 3 which will form Layer 4 × 72,000.

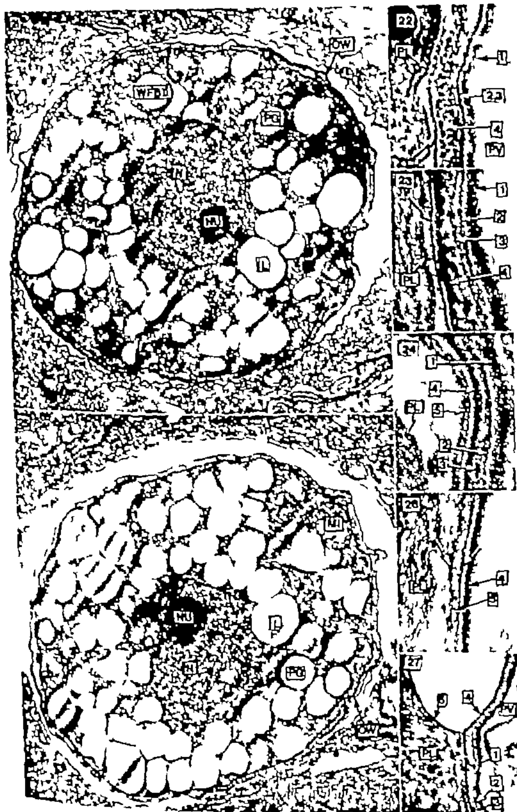
Fig 23 Portion of an organism showing the unit membrane outside the pellicle (arrow) and the particulate matter coalescing to form Layer 4 [illegible] Layers 1 2 and 3 outside Layer 4 × 140,000.

Fig 24 Portion of an organism similar to that in Fig. 23 showing Layers 1 2 3 4 and 5 formed outside the pellicle. × 100 000

Fig 25 Section through an oocyst showing the nucleus, nucleolus, mitochondria, polysaccharide granules and lipid globules contained within the oocyst wall. Note the absence of both WFB I and II. × 12 700

Fig 26 A high magnification of Layers 4 and 5 showing that Layer 5 is limited by unit membranes (arrows) × 180,000

Fig 27 Section through part of an organism at a later stage than that in Fig 25 in which proper polymerisation of the Araldite has not taken place. Note that this effect has occurred between Layers 4 and 5 × 45,000



OW
WFB
PG
N
NU
L
PL
PV

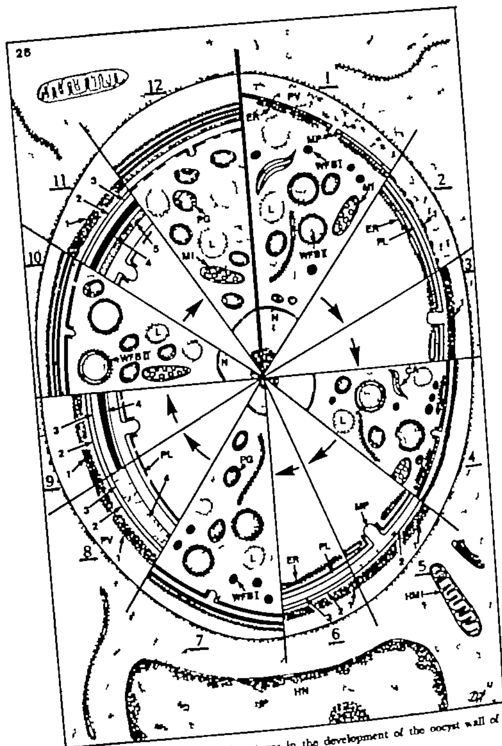


Fig 28 A diagram representing progressive stages in the development of the oocyst wall of *Toxoplasma gondii*.

by *Michel* (1974) and *Porchet Hennere & Omiöres* (1973) respectively Their signif icance in *Toxoplasma* is unknown but they are peculiar to the macrogamete. Fingerlike protrusions of the nucleus has been reported in *Eimeria maxima* by *Mehlhorn* (1972) and material from the nucleus has been reported in the cytoplasm (*Scholtyseck* 1963 b) but their relationship to the double-membraned vacuoles found in this study is unknown.

The fertilisation of the macrogamete of *Eimeria spp* has only been observed once (*Scholtyseck & Hammond* 1970) In this case it occurred while the macrogamete was within the host cell. It would appear that in *Toxoplasma* fertilisation of the macrogamete must also occur within the host cell, because oocyst formation takes place before the organism enters the lumen. It is improbable that a microgamete could enter through the oocyst wall, which does not appear to possess any special adaptation to facilitate this process, such as a micropyle.

No change in the nuclear structure was observed during oocyst wall formation, although the nucleus now probably represents a combination of both the male and female elements.

The appearance of particulate matter which forms Layer 1 of the oocyst wall in the parasitophorous vacuole is a feature previously unreported for *Toxoplasma* or related organisms. The origin of this material is still unknown but no structural changes were observed in the cytoplasm of the macrogamete. The formation of two unit membranes which are termed Layers 2 & 3 is also peculiar to *Toxoplasma* It is possible that the membranes of rough endoplasmic reticulum, found close to the pellicle at this time, are involved in the formation of Layers 1 2 and 3

The formation of Layers 4 & 5 with the apparent involvement of the WFB I and WFB II is similar to that reported for the two layered oocyst wall of *Eimeria spp* (*Scholtyseck* 1963 a *Scholtyseck & Voigt* 1964 *Scholtyseck et al* 1969 b *McLaren* 1969 *Sénaud & Cerná* 1970 *Dubremetz & Yvore* 1971 *Mehlhorn* 1972) The staining properties of these two layers are also similar to that of *Toxoplasma* and from this it would appear that Layers 4 & 5 in *Toxoplasma* are homologous with the outer and inner layers of the oocyst wall of *Eimeria spp* The appearance of granular material which coalesces to form Layer 4 is similar to that reported for the outer layer of *Eimeria necatrix* (*Dubremetz & Yvore* 1971) The one difference in the method of formation of these layers between *Eimeria spp* and *Toxoplasma* is that in *Eimeria spp* the wall forming bodies coalesce within the limiting membrane of the organism to form these two layers (*Scholtyseck et al* 1971 b) but in *Toxoplasma* these layers form outside the intact pellicle of the original macrogamete. The two layers of the oocyst wall of *Eimeria spp* are limited by unit membranes (*Scholtyseck* 1973) In our study it was possible to observe unit membranes limiting Layer 5 but due to the osmiophilic nature of Layer 4 it was impossible to tell if it was limited by unit membranes.

The ultrastructure of the oocyst wall of *Eimeria spp* (*Roberts et al.* 1970) and *Isospora canis* (*Speer et al* 1973 a) observed

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Fig. 28 (cont.)
The first evidence of oocyst formation is the appearance of particulate matter in the parasitophorous vacuole, No 1 This particulate matter precipitates to form Layer 1 of the oocyst wall, Nos. 2 and 3 The organism still contains all the organelles found in the macrogamete, No. 4 The unit membranes which constitute Layers 2 and 3 of the oocyst wall are formed between Layer 1 and the pellicle, Nos. 5 and 6. During this development, membranes of rough endoplasmic reticulum are present below the pellicle the organism still contains all the organelles found in the macrogamete, No. 7 A unit membrane then forms between Layer 3 and the pellicle between this membrane and Layer 3 is found particulate matter which coalesces to form Layer 4 of the oocyst wall Nos 8 and 9. At this stage it is found that the wall-forming bodies of Type I have disappeared from the cytoplasm of the organism, No. 10. Layer 5 of the oocyst wall is formed between Layer 4 and the pellicle (No. 11) and this is accompanied by the disappearance of the wall-forming bodies of Type II the cytoplasm is filled with polysaccharide granules and lipid droplets, No. 12.

after excystation was similar to that of the *Eimeria spp* observed at the time of formation (*Scholtyseck et al* 1971 b) but whether *Toxoplasma* retains its five layered oocyst wall throughout development must await further research

The oocyst wall of *Toxoplasma* differs from that reported for both *in vivo* and *in vitro* studies of *Sarcocystis sp* (*Mehlhorn & Scholtyseck* 1974 *Vetterling et al* 1973) In these cases only one layer was reported although in the *in vitro* study numerous unit membranes were observed below it. It was also noted that only one type of wall forming body was present in the macrogamete (*Vetterling et al* 1973) and it is possible that these observations are related.

Membranes outside the two-layered oocyst wall have been reported in *Eimeria spp* by *McLaren* (1969) *Dubremetz & Yvore* (1971) and *Lee & Millard* (1971) These were reported as being the remains of the limiting membrane of the macrogamete and therefore differ from the Layers 1 2 & 3 reported in this study which were formed in sequence outside the pellicle.

The change occurring in the oocyst which prevents proper penetration and polymerisation of the embedding material is similar to that found in oocysts in the faeces and it would appear that the resistant nature of the oocyst wall develops prior to being shed into the lumen.

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REFERENCES

Colley F C., Fine structure of microgametocytes and macrogametes of *Eimeri nieschulzi*. J Protozool. *14* 663–674 1967

Colley F C & Zaman V. Observations on the endogenous stages of *Toxoplasma gondii* in the cat ileum. II Electron microscope study South East Asian J trop. Med. Pub. Hlth. *1* 465–480 1970

Dubremetz J F & Yvore P., Elaboration de la coque oocystique chez la Coccidie *Eimeria necatrix* Johnston 1970 (Sporozoaires, Coccidiomorphes) Étude au microscope électronique. C. R. Soc. Biol. (Paris) *165* 862–866, 1971

Ferguson, D J P., Hutchison W M Dunachie J F & Siim J Chr Ultrastructural study of early stages of asexual multiplication and microgametogony of *Toxoplasma gondii* in the small intestine of the cat Acta path. microbiol. scand. Sect. B, *82* 167–181 1974

Frenkel J K. Dubey J P & Miller N L Toxoplasma gondii in cat Faecal stages identified as coccidian oocysts Science *167* 893–896, 1970.

Hoare C A. The developmental stages of *Toxoplasma*. J trop. Med Hyg. *75* 56–58, 1972.

Hutchison W M., Dunachie J F Siim J Chr & Work K Life cycle of *Toxoplasma gondii*. Br med. J *4* 806 1969

Hutchison W M Dunachie J F Siim J Chr & Work K Coccidian-like nature of *Toxoplasma gondii*. Br med. J *1* 142–144 1970.

Hutchison W M., Dunachie J F Work, K & Siim J Chr The life cycle of the coccidian parasite *Toxoplasma gondii* in the domestic cat. Trans. roy Soc trop. Med. Hyg. *65* 380–399 1971

Lee D L. & Millard B J The structure and development of the macrogamete and oocyst of *Eimeria acervulina* Parasitology *62* 31 34 1971

McLaren, D J Observations on the fine structural changes associated with schizogony and gametogony in *Eimeria tenella*. Parasitology *59* 563–574 1969

Mehlhorn H Elektronenmikroskopische Untersuchungen an Entwicklungsstadien von *Eimeria maxima* (Sporozoa, Coccidia) I Die Feinstruktur der Makrogameten. Z Parasitenk *39* 161–182, 1972

Mehlhorn H & Scholtyseck E. Licht und Elektronenmikroskopische Untersuchungen an Entwicklungsstadien von *Sarcocystis tenella* aus der Darmwand der Hauskatze I Die Oocysten und Sporocysten Z. Parasitenk. *43* 251–270, 1974

Michael, E. Ultrastructural aspects of the development of the tissue stages of *Eimeria acervulina*. Proceedings British Society for Parasitology 2-4 April 1974 Parasitology 69 XIX, 1974

Overdulve J P The identity of *Toxoplasma* Nicolle & Manceaux, 1909 with *Isospora* Schneider 1881 (I) Koninkl. Nederl. Akad. van Wetenschappen (Amsterdam) Proc., Series C 73: 368–378, 1970.

Pelster B Vergleichende elektronenmikroskopische Untersuchungen an den Makrogameten von *Isospora felis* und *I. rivolta*. Z. Parasitenk. 41 29–46, 1973.

Pelster B. & Piekarski, G., Elektronenmikroskopische Analyse der Mikrogametenentwicklung bei *Toxoplasma gondii*. Z. Parasitenk. 37 267–277 1971

Pelster B & Piekarski G: Untersuchungen zur Feinstruktur des Makrogameten von *Toxoplasma gondii*. Z. Parasitenk. 39 225–232 1972.

Piekarski, G Pelster B. & Witt H M., Endopolygenie bei *Toxoplasma gondii*. Z. Parasitenk. 36 122 130 1971

Piekarski G & Witte H M., Der Erreger der Toxoplasmose—ein spezifischer Katzenparasit. Umschau. Heft 11 342–343 1970

Porchet-Henneré E. & Orneldres R., Étude ultrastructurale des stades "Hepatiques" (Gamontes) de *Grasséella microcosmi* Coccidie parasite du tunicier *Pyura microcosmus*. Protistologica IX 107 211 1973

Roberts, W L., Speer C A. & Hammond D M Electron and light microscope studies of the oocyst walls, sporocysts, and excysting sporozoites of *Eimeria callospermophili* and *E. larimerensis* J Parasit. 56 918–926, 1970.

Scholtyseck E. Über die Feinstruktur von *Eimeria perforans* (Sporozoa) Z. Parasitenk. 22 123–132, 1962.

Scholtyseck E. Elektronenmikroskopische Untersuchungen über die Wechselwirkung zwischen dem Zellparasiten *Eimeria perforans* und seiner Wirtszelle Z. Zellforsch. 61 220–230 1963 a.

Scholtyseck E Vergleichende Untersuchungen über die Kernverhältnisse und das Wachstum bei Coccidiomorphen unter besonderer Berücksichtigung von *Eimeria maxima*. Z. Parasitenk. 22 428–474 1963 b.

Scholtyseck E Ultrastructure. In Hammond, D. M & Long, P L. (Eds): Coccidia. University Park Press, Baltimore 1973 pp. 81–144

Scholtyseck E Gönnert R. & Haberkorn A Die Feinstruktur der Makrogameten des Hühnercoccids *Eimeria tenella* Z. Parasitenk. 33 31 43 1969 a.

Scholtyseck E. & Hammond D M., Electron microscope studies of macrogametes and fertilization in *Eimeria bovis*. Z. Parasitenk. 34 310–318, 1970.

Scholtyseck E. Hammond D M & Ernst J V., Fine structure of the macrogametes of *Eimeria perforans* *E. stiedae* *E. bovis* and *E. auburnensis* J Parasit. 52 975–987 1966.

Scholtyseck E., Mehlhorn H & Haberkorn, A., Die Feinstruktur der Makrogameten des Mause coccids *Eimeria falciformis*. Z. Parasitenk. 37 44–54 1971 a.

Scholtyseck E., Mehlhorn H & Hammond D M Fine structure of macrogametes and oocyst of Coccidia and related organisms. Z. Parasitenk. 37 1–43 1971 b.

Scholtyseck E., Rommel, A & Heller G., Licht und elektronenmikroskopische Untersuchungen zur Bildung der Oocystenhülle bei Eimerien (*Eimeria perforans* *E. stiedae* und *E. tenella*) Z. Parasitenk. 31 289–298, 1969 b.

Scholtyseck E & Voigt W H Die Bildung der Oocystenhülle bei *Eimeria perforans* (Sporozoa) Z. Zellforsch. 62 79–292, 1964

Sebastianova V G Some data on the oocyst sheath formation in *Eimeria tenella* (Sporozoa, Eimeriidae) Tsitologiya. 12 238–242, 1970 (In Russian)

Sheffield H G., Schizogony in *Toxoplasma gondii*. An electron microscope study Proc. helminth. Soc. Wash. 37 237–242 1970.

Sheffield H G & Melton M L. *Toxoplasma gondii*. The oocyst, sporozoite and infection of culture cells. Science 167 892–893 1970.

Siim J Chr Hutchison W M & Work K Transmission of *Toxoplasma gondii*. Further studies on the morphology of the cystic form in cat faeces. Acta path. microbiol. scand. 77 756–757 1969

Speer C A Hammond, D M Mahrt J L & Roberts W L., Structure of the oocyst and sporocyst walls and excystation of sporozoites of *Isospora canis*. J Parasit. 59 35–41 1973

Speer C A Hammond D M Youssef N N & Danforth H D Fine structural aspects of macrogametogenesis in *Eimeria magna*. J Protozool 20 274–281 1973 b.

Vetterling, J M., Pacheco N D & Fayer R. Fine structure of gametogony and oocyst formation in *Sarcocystis sp* in cell culture. J Protozool. 20 613–621 1973.

Vivier E & Petitprez, A., Données ultrastructurales complémentaires morphologiques et cytochimiques sur *Toxoplasma gondii*. Protistologica VIII 199–221 1972

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SUBTYPING OF HERPES SIMPLEX VIRUS

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Vahlne A., Blomberg, J., Olofsson, S. & Lycke, E. Subtyping of herpes simplex virus. Acta path. microbiol. scand. Sect. B 83 506-512, 1975

A number of different techniques to be used for the subtyping of herpes simplex virus (HSV) strains were studied. The strains were inoculated on chorio-allantoic membranes of embryonated eggs and on green monkey kidney (GMK) cells in order that the morphology of plaques produced might be observed. They were classified serologically by determination of K-values and inoculated intracerebrally in mice in order that their pathogenicity for mice might be observed. The inhibitory effect of high concentrations of thymidine on the multiplication of the strains in GMK cells cultures and the heat-stability of the virus-induced thymidine kinases were investigated. Rates of inactivation of the strains in the presence of $AgNO_3$ were compared and, finally the association of focal liver necrosis in intraperitoneally inoculated mice with the results of the serological typing was observed. The results suggested that the liver necrosis test was simple as well as accurate and useful as a screening typing-test. Reliable results were also obtained serologically and by the method demonstrating differences in the heat-stability of viral thymidine kinase Using the other methods studied, difficulties to obtain clear-cut or reproducible typing results were encountered.

Key words Herpes simplex virus subtyping

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The two subtypes of herpes simplex virus (HSV) differ in antigenic composition (5 16, 23 26) and in several other biological (11 20 24) biochemical (9 12) and physical (8) properties. Nevertheless, subtyping of HSV might be difficult since type-differentiating characteristics are weak in relation to markers common of the subtypes. Classification of strains of HSV into types 1 and 2 can therefore seldom be based on one kind of test only and information to be gained by different methods is required. In the present study various methods which according to the available reports may discriminate between the two HSV subtypes were investigated. The reliability of the methods as well as their usefulness as screening tests were considered.

MATERIAL AND METHODS

Viruses Three strains, two type 1 strains (F and MP) and one type 2 strain (G) were kindly supplied by Dr B. Roizman Chicago; five type 2 strains (6503 7875 16516, B4327 UR, B4181 CX) were obtained from Dr S Jeansson, Stockholm eight strains (GB, GE, TN MA, KJ 502, KJ 505 FB and AO) were isolated in our laboratory All the strains studied were plaque-purified.

Cell culture A green monkey kidney (GMK) cell line was cultured at 36 C in 5 cm wide plastic petri dishes using Eagle medium (MEM) supplemented with calf serum and antibiotics. For plaquing of the virus the same medium as described

above as used, but containing in addition 1 per cent methylcellulose. The cultures were incubated at 36 C and the plaques were registered on the fourth day after virus inoculation.

Serological typing. K-values were determined according to *Wildy* (27). Virus and serum mixtures were incubated for 10, 20 and 30 min in ice-bath and remaining infective virus was plaque-titrated in GMK cell cultures. The F (type 1) and G (type 2) strains were employed for the preparation of HSV antisera used in the tests. The cytoplasmic fraction of HSV infected rabbit kidney cells was centrifuged on a dextran T70 gradient (11–38 per cent) at 41,000 × g for 2 hours. The banded virions were inoculated into the conjunctivae of both eyes of the rabbit. Three weeks later, when the acute conjunctivitis had passed off, 0.1 ml of the same material of banded virions were injected intramuscularly (i.m.). The i.m. injections were repeated every 3 weeks and the rabbits were bled after 6 months. The two antisera used for serological typing of HSV strains showed homologous titres in complement fixation and neutralisation tests of 256 and 128–256, respectively. The heterologous neutralization titres were about 10 times lower. In determinations of K-values, the sera were used in a dilution of 1/10 and mixed with an equal volume of the particular virus strain studied containing 4–5 $\log_{10}$ units of pf per ml.

Inhibition of virus multiplication by thymidine. GMK cell cultures in plastic petri dishes, were incubated with 0.5–2 mM thymidine for 24 h and then inoculated with virus. The same concentration of thymidine was added to the Eagle overlay medium. The cells were read on day 3.

Assay of thymidine kinase activity. Enzyme preparation was carried out according to the method described by *Brunick & R pp* (1). GMK-cells were infected by 5–10 PFU per cell. After 18 hours incubation at 37 C, the cells were washed twice with a buffer (pH 8.0) containing 0.05 M KCl and 5 mM 2-mercaptoethanol. The cells were harvested by scraping with rubber policeman, pelleted and suspended in 0.25 ml of the same buffer.

The suspension was sonicated and centrifuged for 60 minutes at 19,000 rev/min in the Spinco 60 Ti rotor. The resulting supernatant was used as enzyme preparation in the thymidine kinase tests. The enzyme reaction was carried out in microtiter plates at pH 8.0 and the reaction mixture, 75 µl, consisted of 5 mM ATP, 5 mM $MgCl_2$, 0.05 M Tris, H-thymidine (2.5 µCi, spec. activity 5000 mCi/mmol) and 25 µl of the enzyme preparation.

The reaction was performed at 37 C for 20 minutes and was stopped by chilling the microtiter plates in an ice bath. 20 µl of the reaction mixture was then placed on a 20 × 20 cm thin layer chromatography plate covered with 0.25 mm of silica gel. Thymidine and phosphorylated thymidine were then separated by dichloromethane : methanol : water (50:50:4). This solvent gave a separation of nucleoside from nucleotide. Detection of radioactivity was carried out either by scraping the adsorbent into counting vessels for liquid scintillation or by scanning the plates by means of a thin layer radioactivity scanner (Berthold Friesecke, Dünnschichtscanner II). Enzyme activity was calculated as pmoles phosphorylated thymidine in the standard mixture per 25 µl enzyme per 20 minutes.

Inactivation of virus with $AgNO_3$. The inactivation was performed according to *Coleman et al.* (3). Briefly $AgNO_3$ was added to the virus suspensions to a final concentration of 40 µM of $AgNO_3$ and were kept in a waterbath at 37 C. However, the actual Ag^+ concentration became much lower due to a presence of Cl^- in the cell culture medium. After varying times of incubation, samples were taken, immediately diluted in Hanks BSS and titrated for residual infectivity in GMK cell cultures.

Induction of liver necrosis in mice. Swiss albino mice, three weeks old, were injected intraperitoneally with 0.1 ml of virus suspension. After various times of incubation, the mice were sacrificed and the livers examined.

RESULTS

K-values, plaque morphology and mouse pathogenicity. The K values listed in Table 1 indicated that 7 out of the 8 untyped strains should be classified as type 1 while one was serologically related to the type 2 strains. This latter strain was isolated from a genital tract infection.

The morphology of plaques produced on chorio-allantoic membranes (CAM) of embryonated eggs or on GMK cells varied. Large plaques on CAM were associated with type 2 or genital strains only, but two of the type 2 strains studied caused small plaques exclusively. There seemed neither to be any association between serological type and size of plaques observed on the GMK cells. Although large plaques were seen only in the case of the type 1 strains, strains producing small plaques were found among both subtypes.

The pathogenicity of the strains for intracerebrally inoculated 3-weeks-old mice seemed not to be a particularly type-related character. Strains highly pathogenic for mice were found among strains serologically related to the type 1 as well as type 2 strains.

TABLE 1 *Plaque Morphology Mouse Pathogenicity and the Serological Type-Relationship of HSV Strains Studied*

Strain	Subtype or origin	Plaque morphology on		No pfu/LD_{50}	K-values of	
		CAM	GMK		Type 1 serum	Type 2 serum
F	1	small	small	ND	0.74	0 07
MP	1	small	large	ND	1.81	1 41
G	2	small	small	ND	2.16	3.68
6503	2	small	small	ND	2.14	4.77
7875	2	large	small	ND	0.48	2.55
16516	2	large	small	ND	1.31	2.33
B 4327 UR	2	large	small	ND	ND	ND
B 4181 CX	2	mixed	small	ND	0.48	1 17
CB	neonatal	small	small	2	2.86	1.82
GE	oral	small	small	4	1 41	0.61
TN	neonatal	small	small	10	2.00	0.97
MA	genital	small	small	6	3.39	.24
KJ 502	oral	small	large	1	4.11	2.72
KJ 503	oral	small	large	0.2	0.69	0.13
AO	genital	mixed	small	1	0 51	0 81
FB	neonatal	small	small	2	1.89	0.20

ND = not done.

Inhibition of virus multiplication by thymidine The plaque forming capacity of 5 HSV strains (F KJ 503 and TN classified as type 1 strains and two type 2 strains G and AO) was studied (Table 2) The type 1 strain, F was most resistant to an increased concentration of thymidine of the cell culture medium while the AO strain isolated from a genital tract infection, appeared to be the one most sensitive. In the presence of 2 mM of thymidine, a reduction of 2.6 log units of pfu was encountered in the case of strain AO The three other strains observed, including the type 2 strain, G demonstrated intermediate grades of sensitivity Thus, a reduction in plaque forming capacity of about one log unit was noted in the cases of strains G and KJ 503 whereas the reduction seen in the case of strain TN was 1.5 log units of pfu. Thus, strains, which serologically were of both subtypes, were apparently affected by high concentrations of thymidine in the cell culture medium.

Heat stability of thymidine kinases of HSV types 1 and 2 GMK cells were infected with HSV type 1 strains (F MP and TN) or type 2 strains (G B 4181 Cx and 6503) and enzyme preparations were produced. 0.25 ml of the preparations were incubated at 40 C for 0 5 and 12 minutes, respectively and thymidine kinase activity was assayed. The reduction of the enzyme activity after a heat-treatment of varying length could thus be observed.

Incubation of the type 1 enzyme preparations at 40 C seemed to reduce the thymidine kinase activity only insignificantly or not at all. A maximal reduction from 450 pmoles to 390 pmoles was observed. On the other hand, the type 2 induced enzymes were mark

TABLE 2. *Plaque Formation of HSV in the Presence of High Concentrations of Thymidine Titres Expressed in Log Units of pfu*

Strain	Concentration of thymidine in mM				
	0	0.5	1 0	1.5	2.0
F	4 48	4 18	4.32	4.28	4.32
G	6.16	5.38	5.08	5.14	5.34
KJ 503	6 29	5 60	5 46	5.36	5.33
AO	4 92	3.20	2.78	3.32	2.30
TN	5 64	5.09	5 17	4.32	4 13

ENZYME ACTIVITY IN PER CENT

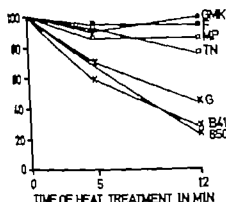


Fig. 1 Thymidine kinase activity after incubation at 37° C for varying periods of time. The results were obtained by uninfected GMK cells (GMK) as well as by cells infected with three HSV type 1 (F MP TN) or three type 2 strains (G B 4181 Cx, 6503)

edly less stable (Fig 1) The minimal reduction noted was from 250 pmoles to 70 pmoles.

Thymidine kinase activity of uninfected GMK cells was resistant to the heat treatment. Loss of enzyme activity in type 2 virus infected preparations of GMK cells was therefore considered to reflect also the effect of the infection on the thymidine kinase activity of the GMK cells. Obviously the thymidine kinase activity of the host cells ceased as a result of the infection.

Inactivation of HSV strains by $AgNO_3$ Suspensions of HSV strains of both subtypes (5 to 6 log units of pfu per ml) were made up to 40 µM of $AgNO_3$. After varying periods of time at 37 C the residual concentrations of virus infectivity were determined.

There seemed to be a trend towards a more rapid inactivation among type 1 strains than among the type 2 strains (Table 3) However the differences observed were in some cases small and varying rates of inactivation were demonstrable also if one and same strain of HSV were used. Sometimes the inactivation of the same strain differed from one experiments to another by as much as 2.5 log units.

TABLE 3. *Effect of Suspending HSV in a Solution of 40 microM of $AgNO_3$ for 10 Min at 37° C Designation / Strains and the Reduction of Infectivity in Log pfu Values are Listed*

Strains serologically classified as			
Type 1		Type 2	
Designation	Reduction	Designation	Reduction
F	1.00	G	0.30
MP	1 19	AO	0.98
TN	3 14	6503	0.63
KJ 503	1 76	7875	0 45
GE	0.97	16516	0.67
MA	2.40		

TABLE 4 *Number of Mice Demonstrating Liver Necrosis after Intraperitoneal Inoculation with 5.0 Log Units of pfu*

Strains serologically classified as			
Type 1		Type 2	
Designation	Results	Designation	Results
F	0/18	G	10/18
MP	0/18	6503	15/18
CB	0/18	7875	10/18
GE	0/18	16516	16/18
TN	0/18	B 4527 UR	17/18
MA	0/18	B 4181 CX	14/18
KJ 502	0/18	AO	8/18
KJ 503	1/18		

Foci of liver necrosis after intraperitoneal inoculation of HSV into mice Mice, in groups of 18, were injected intraperitoneally with the HSV strains studied. Each strain was used in a concentration of 10^6 pfu/ml. On the 3rd–5th day after inoculation, the mice were killed and the livers inspected for macroscopically detectable foci on the liver surface. Each time, 6 mice were observed. The results are summarized in Table 4

Several small but distinctly marked white spots of necrotic tissue were seen on the liver surface in mice inoculated with the established type 2 strains studied. None of the established type 1 strains produced such necrotic plaques. The genital strain AO which according to the serological test as well as the thymidine test apparently belonged to sub-

type 2, produced several easily detectable foci of liver necrosis. The other strain (MA) isolated from a genital tract infection did not. Serologically this latter strain was also related to a higher degree to the type 1 than to the type 2 strains. Only one of the strains (KJ 503) which according to the serological test, belonged to type 1 caused occasionally a few necrotic liver foci. These foci were diffuse, much smaller and easily distinguishable from those observed in mice inoculated with the established type 2 strains.

DISCUSSION

Interest in the subtyping of HSV is growing This is partly due to the fact that observations indicating that the type 1 and 2 viruses are responsible for different clinical syndromes are accumulating, but it emerges also from the discussion about a possible importance of HSV infections in the pathogenesis of some diseases of unknown aetiology (15 19 25)

Studies of base sequences of HSV type 1 and 2 genomes have revealed that the two subtypes differ by as much as 50 per cent (9 12) In spite of the genetic differences, however any pronounced subtype characteristics are non-existed. The typing may consequently be complicated, as for the serological typing, by the masking of subtype differences by strong group-specific reactions (4)

Statements about the type-relation of isolated strains must, as a rule, be based on results obtained by more than one differentiating technique. A serological test is often combined with one or more non-immunological methods. Determination of K-values is the serological technique on which most subtyping of HSV is based (26) The method is laborious, however and is hardly applicable as a screening test. The selection of good, i.e. relatively type-specific, antisera is important and such antisera may be difficult to produce (21) although appropriate immunization techniques recently have been presented (7) Other serological methods such as diffusion-in-gel-techniques (23) counter-electro-osmophoresis (6) and complement fixation tests (22) have also been used.

It has been claimed, particularly in the older literature that plaque-formation by HSV strains on chorio-allantoic membranes (CAM) of embryonated eggs or on different cell cultures can differentiate between type 1 and type 2 strains (10, 15 18) The variety in size of plaques, not only in strains which serologically belong to one and the same type, but also in variants of one and same strain, however has invalidated the plaque morphology tests as a typing procedure. In accordance with this, we found in the present study that the appearance and size of plaques seemed not to be strictly type-associated characteristics although large plaques on CAM were observed only in the case of the established type 2 strains or strains of genital tract origin. *Cohen et al* (2) have observed that thymidine, although a precursor in DNA synthesis, inhibits multiplication of HSV type 2 but not of type 1 strains if added in high concentrations to the culture medium. To see whether this difference possibly could be used as a complementary method for the typing of HSV it was tested on a number of strains. Differences in sensitivity to thymidine between type 1 and 2 strains were observed, but the occurrence of strains of both serological subtypes with an intermediate grade of sensitivity did not suggest that the method was reliable for typing

Thymidine kinase activity is induced in cells infected with HSV The enzyme is a deoxypyrimidine kinase which differs from the host cell enzyme in substrate specificity K_m, allosteric properties, pH optimum, immunological specificity and temperature sensitivity The enzymes induced by HSV subtypes 1 and 2 respectively differ in immunological specificity allosteric properties and temperature sensitivity (17) These differences can be exploited and used for subtyping of HSV isolates by which thymidine kinase activity by a modified method, may be tested.

Thymidine kinase provided by cells infected with HSV type 1 exhibited a signifi-

cantly higher resistance to heat than the corresponding enzymes from cells infected by HSV type 2. This is in accordance with the results obtained by *Ogino & Rapp* (17). The fact that destruction of the HSV 1 enzyme was accelerated at temperatures slightly above 40° C as well as during incubation for more than 15–20 min, however, brings about that the method is sensitive to technical disturbances. Taking these observations into account we consider still the method valuable as a means by which the typing of selected strains can be confirmed.

One of the tests studied has been reported to reveal type-related differences demonstrable by different sensitivity to the inactivation with AgNO of types 1 and 2 strains (3). In our hands, this test revealed unquestionable difference between the two subtypes. The disadvantages were the ocurrence of strains of intermediate sensitivity and the difficulty to obtain reproducible results, the latter being most disturbing.

The demonstration of necrotic foci on the liver surface in intraperitoneally inoculated mice is a simple method described by *Mogensen et al.* (14). The method gave reproducible results if standardized inocula were used. Strains serologically behaving as type 2 strains produced focal liver necroses while such necroses were not regular findings after inoculation of type 1 strains. Only one strain which serologically seemed to belong to subtype 1 caused occasionally single necrotic liver foci. As the method is easy to perform and there seemed to be a good correlation with results obtained by serological typing, the method seems useful, particularly as a method by which to screen isolated strains of HSV.

Our results would suggest that three of the tests studied could be particularly useful in the typing of isolated HSV strains. If used as a screening test, mice inoculated intraperitoneally should be observed for the occurrence of focal liver necroses. This preliminary subtyping of strains could be confirmed serologically and, in cases where no clear-cut results were obtained serologically, the required additional information could be achieved by studies of the heat-stability of the virus induced thymidine kinases.

This study was supported by the *Swedish Medical Research Council*, grant No. B75-16X-4314-01 and by grants from the *Medical Faculty, University of Göteborg*. We are indebted to Miss *Birgitta Thoreson* and Miss *Lena Stenborg* for skillful technical assistance.

REFERENCES

1. *Bresnick, E. & Rapp, F.*: Thymidine kinase activity in cells abortively and productively infected with human adenoviruses. Virology *34*: 799–802, 1968.
2. *Cohen, G. H., Factor, M. N. & Ponce de Leon, M.*: Inhibition of herpes simplex virus type 2 replication by thymidine. J. Virology *14*: 20–25, 1974.
3. *Coleman, V. R., Wilkie, J., Levinson, W. E., Stevens, T. & Jawetz, E.*: Inactivation of herpesvirus hominis types 1 and 2 by silver nitrate in vitro and in vivo. Antimicrob. Ag. Chemother. *4*: 259–262, 1973.
4. *Hamper, B. & Martos, L. M.*: In: Kaplan, A. S. (Ed.): The Herpesviruses. Academic Press, New York and London 1973, pp. 221–259.
5. *Heness, R. W., Powell, K. L., Robinson, D. J., Sim, Caroline & Watson, D. H.*: Type specific and type common antigens in cells infected with herpes simplex virus type 1 and on the surfaces of naked and enveloped particles of the virus. J. gen. Virol. *22*: 159–169, 1974.
6. *Jeansson, S.*: Differentiation between herpes simplex virus type 1 and type 2 strains by immunoelectroosmophoresis. Appl. Microbiol. *24*: 96–100, 1972.
7. *Jeansson, S.*: Production of herpes simplex antisera with increased type specificity in guinea pigs by antibody suppression. Proc. Soc. Exp. Biol. Med. *147*: 788–794, 1974.
8. *Kieff, E. D., Bachenheimer, S. L. & Roizman, B.*: Size, composition and structure of the deoxyribonucleic acid of herpes simplex virus subtypes 1 and 2. J. Virology *8*: 125–132, 1971.
9. *Kieff, E., Hoyer, B., Bachenheimer, S. & Roizman, B.*: Genetic relatedness of type 1 and type 2 herpes simplex viruses. J. Virology *9*: 738–745, 1972.
10. *Lancz, G. J.*: Herpes simplex viruses types 1 and 2: type and strain specific characteristics affecting virus plaque formation. Arch. ges. Virusforsch. *46*: 36–43, 1974.
11. *Longson, M.*: A temperature marker test for the differentiation of strains of herpes virus hominis. Ann. Inst. Pasteur *120*: 699–706, 1971.

12 *Ludwig, H O., Biswal N & Benyesh-Melnick M.:* Studies on the relatedness of herpesviruses through DNA DNA hybridization. Virology *49* 95–101 1972.

13 *Lycke E. Norrby R. & Roos B E.:* A serological study on mentally ill patients. With particular reference to the prevalence of herpes virus infections. Brit. J of Psychiat. *113* 1407–1411 1974

14 *Mogensen S C., Teisner B. & Kessel Andersen H.,* Focal necrotic hepatitis in mice as a biological marker for differentiation of herpes virus hominis type 1 and type 2 J gen. Virol. *25* 151–157 1974

15 *Munk K & Ludwig, G.,* Properties of plaque variants of herpes virus hominis strains of genital origin. Arch. ges. Virusforsch. *37* 308–315 1972.

16. *Nahmias A. J & Dowdle W R.* Antigenic and biologic differences in Herpesvirus hominis. Progr Med. Virology *10* 110–159 1968.

17 *Ogino T & Rapp F.,* Differences in thermal stability of deoxythymidine kinase activity in extracts from cell infected with herpes simplex virus type 1 or type 2. Virology *46* 953–955 1971

18. *Parker J D J & Banatvala J E.,* Herpes genitalis. Clinical and virological studies. Brit. J vener Dis. *43* 212–216 1967

19 *Rimon R. & Halonen P* Herpes simplex virus infection and depressive illness. Dis. nerv Syst. *30* 338–340, 1969

20 *Rodgers, F G.:* Infection, haemorrhage and death of chick embryos after inoculation of herpes simplex virus type 2 or to the chorioallantoic membrane. J gen. Virol. *21* 187–191 1973

21 *Savage T., Roizman B. & Heine J W.:* Immunological specificity of the glycoproteins of herpes simplex virus subtypes 1 and 2. J gen. Virol *17* 31–48, 1972.

22. *Schneweis K. E.,* Serologische Untersuchungen zur Typedifferenzierung des herpes virus hominis. Z. Immunitaetsforsch. *124* 24–28, 1962.

23 *Schneweis K. E. & Nahmias, A. J* Antigens of herpes simplex virus type 1 and 2—immunodiffusion and inhibition passive hemagglutination studies. Z. Immunitaetsforsch. *141* 471–487 1971

24 *Smith J W., Rodriguez J E. & McKee A. P.,* Biological characteristics of cloned populations of herpes simplex virus types 1 and 2. Applied Microbiology *21:* 350–357 1971.

25 Symposium Herpesvirus and cervical cancer. Cancer Res. *33* 1345–1563 1973.

26. *Vestergaard B. F* Crossed immunoelectrophoretic characterization of herpesvirus hominis type 1 and 2 antigens. Acta path. microbiol. scand. Sect. B, *81:* 808–810, 1973.

27 *Wildy P* Herpes virus and antigens. In Biggs, P M., de Thé, G. & Payne, L. N. (Eds.) Oncogenesis and Herpes viruses. Int. Agency Res. Cancer Lyon 1972, pp. 409–419.

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THE EFFECT OF BACITRACIN AND Mn(II)IONS UPON THE PRODUCER STRAIN *BACILLUS LICHENIFORMIS*

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Haavik, H. I. The effect of bacitracin and Mn(II)ions upon the producer strain *Bacillus licheniformis* Acta path. microbiol. scand. Sect. B 83; 513–518, 1975

The peptide antibiotic bacitracin is inhibitory to growth of the producer strain *Bacillus licheniformis* only in the presence of excess manganese(II)ions. Both the early and the late growth are inhibited in a similar manner upon addition of bacitracin and manganese(II)ions. Thus, *B. licheniformis* does not develop resistance to its own antibiotic during growth. Added bacitracin is stimulatory to growth of *B licheniformis* in media with a very low content of manganese(II)ions. These results support the hypothesis that bacitracin participates in the manganese transport of the producer strain *B. licheniformis*.

Key words *Bacillus licheniformis* bacitracin; Mn(II)ions.

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Since many antibiotics are very potent antimicrobial agents, the question as to how the antibiotic producing organisms are able to survive their own abundant production has been given some attention (2, 9) The microbial production of antibiotics is conveniently separated into a growth phase (Trophophase) followed by a production phase (Idiophase) (1 8, 10) During the growth phase producer organisms are usually sensitive to their own antibiotic, whereas during the production phase even large amounts of the antibiotic produced have no effect (2, 9 12) It is therefore suggested that microorganisms can grow and produce antibiotics because the latter are not formed until after growth (2, 9)

We have recently reported, however that the antibiotic bacitracin was produced after rapid growth of *Bacillus licheniformis* due to an incidental inhibitory effect of low pH and organic acids (4 6) The low pH and the acids arose from the metabolism of glucose which usually is present in media for antibiotic production. Thus in media without glucose or when pH was properly controlled, bacitracin production paralleled growth. The bacitracin produced during the early growth phase was not inhibitory to growth of *B licheniformis* (6) This does not support the suggestion that microorganisms can grow and produce antibiotics because the latter are not formed until after growth.

The aim of the present work was to investigate the effect of bacitracin and Mn(II) ions upon the growth of the producer strain *B. licheniformis* ATCC 10716

MATERIALS AND METHODS

Reagents

All reagents used were of analytical grade. Bacitracin was kindly supplied by Mr A. *Ulvand* A/S Apothekernes Laboratorium for Specialpræparater Oslo 2 Norway The purity of the bacitracin used was according to the rules given in Ph.Nord and U.S.P XVIII

Media and Growth Conditions

The chemically defined medium (M2) for growth of *B. licheniformis* ATCC *10716* and conditions for incubation have been described previously (4) The effect of bacitracin upon the early growth was examined by the addition of sterile filtered bacitracin (Millipore) to the incubation flasks just before inoculation. The effect of bacitracin upon later growth was examined by aseptically adding bacitracin to the flasks after 9 hours of incubation. Manganese(II)ions were added to the cultures during growth as sterile solutions, or were included in the medium before autoclaving The inoculum consisted of 1 per cent of an overnight culture in the M2 medium which resulted in an E_{650} of 0.04 when added to the medium in the shaking flasks.

The amounts of Mn(II)ions present in the inoculum may give a contribution to the medium of 0.1 mg $MnSO_4$/l which is negligible as compared to the 10 mg $MnSO_4$/l usually present in the M2 medium. When the bacteria was grown in the M2 medium without Mn(II)ions added, the inoculum was also grown in the same medium. The traces of Mn(II)ions present in this medium was due to impurities from the other ingredients and might amount to about 0 01–0 001 mg Mn(II)ions/l. The amount of Mn(II)ions in the inoculum in this case will be about 1 per cent of the amount in the medium.

Growth

Bacterial growth was measured as extinction at 650 nm using a Spectronic 20 spectrophotometer as described elsewhere (5)

Assay of Bacitracin

The bacitracin used was determined microbiologically by an agar diffusion method described previously (5)

Inactivation of Bacitracin

A bacitracin solution with a known potency (1000 i.u. bacitracin/ml) was heated at 90 C for 48 hours. Upon reanalyzing no antibiotic activity was found.

RESULTS

Fig 1a shows that the addition of 25 i.u. bacitracin/ml or less had no effect upon the early growth of *Bacillus licheniformis* in the M2 medium. The addition of 50 i.u. bacitracin/ml, however resulted in a significant inhibition of growth. By increasing further the amounts of bacitracin, an increasing growth inhibition was observed (Fig 1a)

The inhibitory effect of 50 i.u. bacitracin/ml upon the growth of *B. licheniformis* markedly increased as the amount of manganese(II)ions in the medium was increased (Fig. 1b) In the presence of 200 mg $MnSO_4$/l the early growth was significantly inhibited when only 5 i.u. bacitracin/ml was added to the medium (Fig. 1c) The bacitracin added did not disappear from the medium during the incubation period, an observation also made by others (9) As seen from Fig 1b high amounts of manganese(II)ions were somewhat inhibitory to growth when added alone.

Fig 2 shows that the addition of 50 i.u. bacitracin/ml later in growth had no effect upon the succeeding growth. However when 5 mg MnSO /l was added together with bacitracin late in growth, a marked inhibition was observed. The addition of 5 mg MnSO /l alone resulted in a slight stimulation of growth. When larger amounts of Mn(II)ions were added alone late in growth, lysis of the bacteria occurred. This lytic activity was enhanced by the addition of bacitracin (Fig 2) The amounts of manganese(II)ions which resulted in lysis when added late in growth had no effect upon growth when added to the early growth phase. It was further found that the addition of 50 i.u. bacitracin/ml together with 5 mg $MnSO_4$/l was inhibitory whenever added during growth.

B. licheniformis grew well when no manganese(II)ions were added to the M2 medium. The traces of manganese(II)ions present were apparently sufficient for growth. When bacitracin was added to this medium a significant stimulatory effect upon the early

Fig. 1 Growth of *B. licheniformis* in the M2 medium with the following additions:

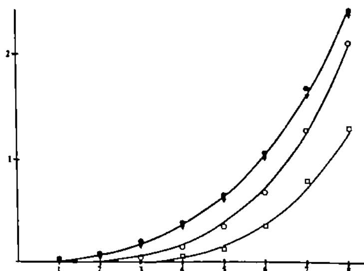


Fig. 1a: no bacitracin added (●) 1, 5, 10, and 25 i.u. bacitracin/ml added (▼) 50 i.u. bacitracin/ml added (○) and 100 i.u. bacitracin/ml added (□)

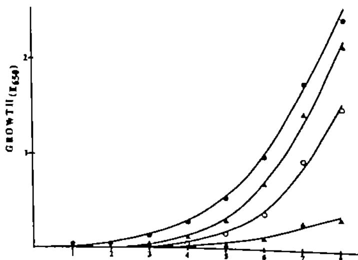


Fig. 1b: 50 mg $MnSO_4$/l added (●) 50 mg $MnSO_4$/l and 50 i.u. bacitracin/ml added (○) 200 mg $MnSO_4$/l added (▲) and 200 mg $MnSO_4$/l and 50 i.u. bacitracin/ml added (△)

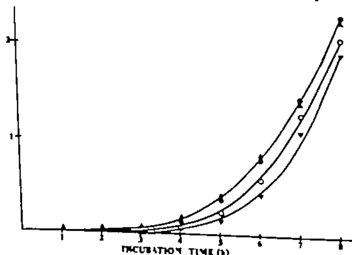


Fig. 1c: 200 mg $MnSO_4$/l added (●) 200 mg $MnSO_4$/l and 1 i.u. bacitracin/ml added (▲) 200 mg $MnSO_4$/l and 5 i.u. bacitracin/ml added (○) and 200 mg $MnSO_4$/l and 10 i.u. bacitracin/ml added (▽)

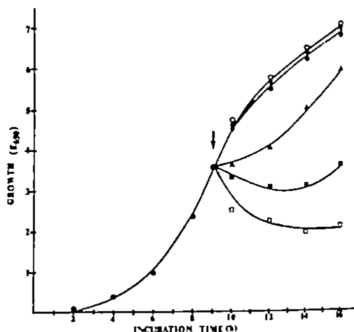


Fig 2 Growth of *B. licheniformis* in the M2 medium with the following additions later in growth. Arrow indicate time of addition: Nothing added (●) 50 i.u. bacitracin/ml added (▼) 5 mg $MnSO_4$/l added (○) 10 mg $MnSO_4$/l added (■) 5 mg $MnSO_4$/l and 50 i.u. bacitracin/ml added (△) and 10 mg $MnSO_4$/l and 50 i.u. bacitracin/ml added (□)

growth was observed (Fig. 3a) The addition of only 2 i.u. bacitracin/ml resulted in the most pronounced stimulation. When the inoculum consisted of spores this was somewhat different. By the addition of spores and bacitracin to the M2 medium with no Mn(II)ions added a marked stimulatory effect upon growth was observed. The addition of 50 i.u. bacitracin/ml resulted in the most pronounced stimulation (Fig 3b) When 50 i.u. bacitracin/ml and 100 mg MnSO /l was added together with the spore inoculum, growth was inhibited (Fig 3b)

When corresponding amounts of inactivated bacitracin was added to bacteria or spores no stimulatory effect upon growth was observed.

DISCUSSION

Our results indicate that the effect of bacitracin upon the growth of the producer strain *Bacillus licheniformis* is dependent upon the amount of manganese(II)ions present in the medium. Both stimulatory inhibitory and lytic effects upon the cells have been observed. In media with a very low content of this cation, bacitracin seems to be stimulatory to growth, whereas in media with a high amount of manganese(II)ions, bacitracin is inhibitory to growth or induces lysis depending upon the amount of bacitracin added.

Snoke & Cornell (9) have previously reported that small amounts of bacitracin markedly inhibited the early growth of *B. licheniformis* ATCC *10716*. They suggested that bacitracin could accumulate during later stages of growth because the inhibitory effect of bacitracin was restricted to the early growth phase only Our results, however show bacitracin is able to inhibit both the early and the late growth of *B. licheniformis* in the presence of manganese(II)ions.

It is further proposed that antibiotic producers can survive their own production because they develop resistance to their own antibiotic during later stages of growth (2, 12) This resistance is suggested to be phenotypic because the growth of these resistant cells in a fresh medium containing the antibiotic, will be inhibited once again (2) Our results, however indicate that *B. licheniformis* does not develop resistance to bacitracin during growth since both the early and the late growth of the organism are in-

Fig. 3. Growth of *B. licheniformis* in the M2 medium with no manganese(II)ions added and with the following additions:

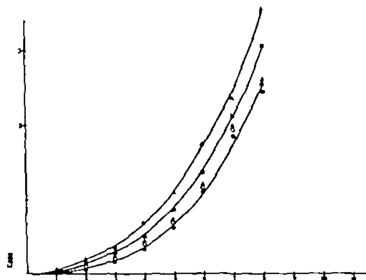


Fig. 3a Bacteria as inoculum with no bacitracin added (●) with 1 i.u. bacitracin/ml added (○) with 2 i.u. bacitracin/ml added (Δ) with 5 i.u. bacitracin/ml added (□) and with 10 i.u. bacitracin/ml added (▲)

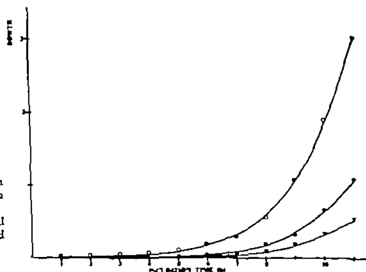


Fig. 3b Spores as inoculum with no bacitracin added (●) with 50 i.u. bacitracin/ml added (○) and with 50 i.u. bacitracin/ml and 100 mg $MnSO_4$/l added (∇)

hibited in a similar manner i.e., *only in the presence of sufficient amount of manganese(II)ions*. It appears that *B. licheniformis* is resistant to its own antibiotic during later stages of growth because there are normally very low concentrations of this cation left in the medium towards end of growth. The addition of 10 mg MnSO /l late in the growth had a marked toxic effect upon the culture whereas the same amounts added to the early growth phase had no such effect. This supports our suggestion, since it is possible that the relatively high amounts of bacitracin produced late in growth (4) act *inhibitorily when sufficient amounts of manganese(II)ions are added.*

Since the activity of many antibiotics is influenced by metal ions (11) it is possible that the frequently observed inhibition of the early growth of producer strains by their own antibiotic may by due to the presence of excess metal ions during early growth. This may explain why "resistant" cells from the production phase are inhibited once again when inoculated in a fresh medium containing the antibiotic.

We have recently put forward the hypothesis that bacitracin may participate in the

transport of manganese(II)ions in *B licheniformis* (7) This hypothesis may explain why bacitracin acts inhibitorily in media with high amounts of manganese(II)ions, and stimulatorily in media with a very low content of the same cation. In the first case too much manganese(II)ions are transported into the cells upon the addition of bacitracin and toxic concentrations are reached. In the latter case bacitracin stimulates growth by increasing the uptake of Mn(II)ions which now are needed by the bacteria.

When bacitracin was added to spores in low Mn(II)ions environments, the stimulatory effect upon growth was more pronounced The spores must probably germinate and grow out before bacitracin is produced. The addition of bacitracin to spores will therefore probably result in a more rapid uptake of sufficient amounts of the desired cation. Since an inactivated bacitracin solution did not stimulate growth the stimulatory effect of bacitracin was not due to impurities present in the bacitracin used

To the authors knowledge this is the first example of a stimulatory effect of an antibiotic upon the producer cells.

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REFERENCES

1. *Demain A. L.:* Regulatory mechanisms and the industrial production of microbial metabolites. Lloydia. *31:* 395–418, 1968.
2. *Demain A L.:* How do antibiotic-producing microorganisms avoid suicide? Annal. N.Y Acad. Sci. *235:* 601–612, 1974
3. *Haavik H I & Thomassen S.:* A bacitracin-negative mutant of *Bacillus licheniformis* which is able to sporulate. J gen. Microbiol. *76* 451–454 1973.
4. *Haavik H I.:* Studies on the formation of bacitracin by *Bacillus licheniformis:* effect of glucose. J gen. Microbiol. *81* 383–390, 1974
5. *Haavik H I.:* Studies on the formation of bacitracin by *Bacillus licheniformis:* effect of inorganic phosphate. J gen. Microbiol. *84* 226–230 *1974*
6. *Haavik, H I.* Studies on the formation of bacitracin by *Bacillus licheniformis* role of catabolite repression and organic acids. J gen. Microbiol. *84* 321–326, 1974.
7. *Haavik H I & Frøyshov Ø.* On the function of peptide antibiotics in the producer organisms. Nature *254* 79–82, 1975.
8. *Sadoff H L.:* Sporulation antibiotics of *Bacillus* species. Spores *5* 157–166, 1972.
9. *Snoke J E. & Cornell N.* Protoplast lysis and inhibition of growth of *Bacillus licheniformis* by bacitracin. J Bacteriol. *89* 415–420 1965
10. *Woodruff H B.* The physiology of antibiotic production the role of the producing organism. Soc. Gen. Microbiol. Symp. *16* 22 46, 1966.
11. *Weinberg E. D* The mutual effects of antimicrobial compounds and metallic cations. Bact. Rev *21* 46–68, 1957
12. *Yoshida, T Weissbach H & Katz, E.* Inhibitory effect of actinomycin upon the producing organism. Arch Biochem. Biophys *114* 252–255 1966

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ON THE FUNCTION OF THE POLYPEPTIDE ANTIBIOTIC BACITRACIN IN THE PRODUCER STRAIN *BACILLUS LICHENIFORMIS*

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The growth of the bacitracin producing strain *Bacillus licheniformis AL* and the bacitracin-negative mutant *SB 319* have been compared at different cultural conditions. Concentrations of the metal chelator EDTA which strongly inhibited the growth of the non-producer only slightly inhibited the growth of the bacitracin producer. The inhibitory effect of EDTA upon *SB 319* was reversed by the addition of excess manganese(II)ions, cobalt(II)ions, or zinc(II) ions to the culture. The addition of several other ions had no such effect. The addition of bacitracin to the EDTA inhibited mutant also promoted growth. When the non-producer was mutated back to bacitracin production, the inhibitory effect of EDTA was lost. It is suggested that bacitracin may normally promote the uptake of several trace metals during growth of the producer organism.

Key words: Bacitracin function; *Bacillus licheniformis*.

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The function of peptide antibiotics in the …ducer organisms is widely unknown (3, …). A strict bacitracin negative mutant has …n isolated from the bacitracin producing …in *Bacillus licheniformis AL* (12, 13) …e mutant and the mother strain have been …pared at different cultural conditions in …der to obtain some information about a …sible function of the peptide antibiotic …itracin. In a previous investigation we …ve shown that the bacitracin-negative …tant may have a markedly less efficient …take mechanism for manganese(II)ions due to lack of bacitracin production (13). The possibility therefore exists that bacitracin may in some manner participate in the metal ion transport of the producer organism.

MATERIALS AND METHODS

Organisms

The bacitracin producing strain *Bacillus licheniformis AL* and the bacitracin-negative mutant *SB 319* were kept as spore suspensions at 4 C throughout the investigation (11).

Media and Growth Conditions

The following complex media were used (Concentrations in g/l distilled water): RM-medium

Bacto-soytone (20) Bacto-peptone (10) $MnSO_4$ H_2O (0.01) RMO-medium: The same composition as the RM-medium except that $MnSO_4$ H_2O was not included. The pH of the media was adjusted to 7 with 1M NaOH before autoclaving at 121 C for 20 min. EDTA or bacitracin were sterile filtered (Millipore) before addition to the cultures. The following salts were added to the RMO-medium as described in the text: $CoCl_2$ $6H_2O$ $CaCl_2$ $2H_2O$ $FeCl_2$ $4H_2O$ $MgCl_2$ $6H_2O$ $ZnCl_2$, $MnCl_2$ $4H_2O$ LiCl, (NH_4) $6Mo_7O_{24}$ $4H_2O$ (25 mg/l). The inoculum consisted of 0.05 ml of the spore suspension. Growth occurred in 500 ml EM-flasks filled with 50 ml medium. The flasks were incubated at 37 C at about 120 cycles/min in a shaking water bath (Model HETO TB SHO2)

Growth

Bacterial growth was measured as extinction at 650 nm (E_{650}) using a Spectronic 20 spectrophotometer

Microbiological Assay of Bacitracin

Bacitracin was determined by an agar diffusion method as described previously (12)

Mutagenic Treatment

The spores of strain *SB 319* were mutated as described elsewhere (12)

Inactivation of Bacitracin

A bacitracin solution with a known potency (1000 i.u. bacitracin/ml) was heated at 90 C for 48 hours. Upon reanalyzing no antibiotic activity was found.

RESULTS

Bacillus licheniformis AL produced about 70 i.u. bacitracin/ml when grown in the RM medium for about 24 h. Bacitracin was pro-

TABLE 1 *Growth and Bacitracin Production by* Bacillus licheniformis AL *in the RM Medium.*

Time of incubation h	Growth E_{650}	Bacitracin production i.u./ml
4	0 2	0.3
8	1.2	0.9
12	3.9	10.5
16	6.0	38
20	7 1	56
24	7.9	68

TABLE 2. *Growth and Bacitracin Production of* Bacillus licheniformis *Strain* AL, *Strain SB 319, and Strain SB 319R in Different Media*

Strain	Medium	Growth (E_{650} at 24 h)	Bacitracin production i.u./ml
AL	RM	8.0	72
AL	RMO	8 1	31
AL	RMO + EDTA	7.9	19
SB 319	RM	7.8	nd
SB 319	RMO	6.9	nd
SB 319	RMO + EDTA	2.0	nd
SB 319R	RM	7.9	55
SB 319R	RMO	7.8	24
SB 319R	RMO + EDTA	7.8	12

nd – not detected.

duced by actively growing cells in this complex medium (Table 1) When manganese-(II)ions were excluded from the medium (RMO-medium) a maximum titre of about 30 i.u. bacitracin/ml was produced (Table 2) This is in agreement with *Weinberg & Tonnis* (26) who reported that manganese was stimulatory to bacitracin production. The mutant *SB 319* showed the same growth rate and about the same maximum crop as *strain AL* when grown in the RM medium, but bacitracin production could not be detected. When *SB 319* was grown in the RMO-medium, maximum crop was somewhat lower than that of *strain AL* (Table 2)

Fig 1 shows that the bacitracin-negative mutant *SB 319* was markedly inhibited by the addition of EDTA to the RMO-medium. The bacitracin producing mother *strain AL* was only slightly inhibited. Maximum bacitracin production by *strain AL* was about 20 i.u. bacitracin/ml when EDTA was a constituent of the medium (Table 2)

Since EDTA is a strong metal chelator it is reasonable to assume that the inhibitory effect of EDTA was due to metal deficiency Several different trace metals were therefore added to the RMO-medium. The inhibitory effect of EDTA upon *SB 319* was reversed by the addition of excess manganese(II)ions (Fig 2) Table 3 shows that the addition of excess Co(II)ions and Zn(II)ions also re-

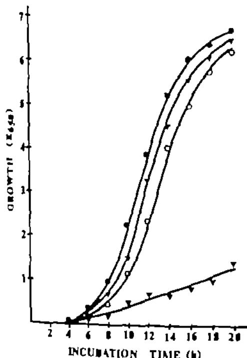


Fig. 1 The effect of EDTA upon growth of *Bacillus licheniformis strain AL* and strain *SB 319* in the RMO-medium. Growth of *strain AL* with no EDTA added (●) and with 100 mg EDTA/l added (O). Growth of *strain SB 319* with no EDTA added (▼) and with 100 mg EDTA/l added (▽)

versed the inhibitory effect of EDTA, whereas several other ions had no such effect.

When bacitracin was added to the EDTA inhibited mutant, growth was also promoted (Fig. 2). A bacitracin solution was inactivated as described under methods. The addition of inactivated bacitracin to the EDTA inhibited mutant had no stimulatory effect upon growth.

The bacitracin-negative mutant was mutated back to bacitracin production by means of UV treatment. One revertant *SB 319R* with a restored bacitracin production was examined further. The revertant of *SB 319* produced about 80 per cent of the bacitracin produced by *strain AL* (Table 2). The bacitracin producing revertant grew as well as the bacitracin producing *strain AL* in the presence of EDTA (Table 2).

DISCUSSION

The first thorough examination of the bacitracin production by *Bacillus licheniformis* was reported by *Hills et al.* (9). They concluded that bacitracin may either be a by-product of metabolism or a product of incomplete metabolism which does not accumulate under conditions most favourable for the producing organism. Since it has now been reported that bacitracin is built up from amino acids by a rather complex enzyme machinery (6), it is unlikely that bacitracin is just a waste product.

Bernlohr & Novelli (1) have proposed that there is a connection between bacitracin production and the sporulation process of *B. licheniformis*. They concluded that bacitracin might be a spore coat component of *B. licheniformis* (2). This hypothesis, how-

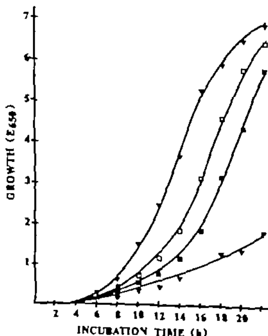


Fig. 2 The effect of EDTA, manganese(II) ions, and bacitracin upon growth of *Bacillus licheniformis SB 319* in the RMO-medium. Growth of *SB 319* with no additions (▼), with 100 mg EDTA/l added (▽), with 100 mg EDTA/l and 25 mg $MnCl_2 \cdot 4H_2O$/l added (□) and with 100 mg EDTA/l and 20 I.u. bacitracin/l added (■)

TABLE 3 *Effect of Excess Metal Ion upon the Growth of the EDTA Inhibited Mutant* SB 319 *in RMO-medium*

EDTA added mg/l	Metal ion added 25 mg/l*	Growth after 14 hours of Incubation – E_{650}
0	non	3.22
100	non	0.70
100	Co(II)	2.00
100	Cu(II)	0.52
100	Fe(II)	0.60
100	Li(I)	0.45
100	Mg(II)	0.47
100	Mo(VI)	0.63
100	Zn(II)	2.23

* As a salt.

ever has not been confirmed by *Snoke* (24) and *Marschke & Bernlohr* (18). Generally the formation of most peptide antibiotics has been associated with the sporulation process of the producer organisms (1, 4, 10). Recently it has been suggested that peptide antibiotics may control some of the early events in sporulation (20, 22, 23). As for mycobacillin produced by *Bacillus subtilis* (19), bacitracin produced by *Bacillus licheniformis* (12) and gramicidin S produced by *Bacillus brevis* (15), mutants have been isolated which produce no antibiotic but still sporulate. This rules out a direct role for these peptide antibiotics in the sporulation process of the producer strains (21). The possibility that bacitracin could accumulate as a result of hydrolysis of cell wall material during the early stages of sporulation has also been mentioned (1). A de novo synthesis of bacitracin from amino acids in a cell free preparation of *B. licheniformis* however has been reported (7, 14).

Weinberg (25) has proposed that bacitracin may participate in the induction of the lysis of vegetative cells that occurs during the early stages of sporulation. This proposal is mainly based on the observations that a) bacitracin is mainly produced after growth, b) bacitracin is able to alter the integrity of bacterial cell membranes and can disrupt protoplasts of the producer cells. Our results, however, show that *B. licheniformis* produces bacitracin during growth in the complex RM medium. In a previous investigation (11) bacitracin was also produced during active growth in a synthetic medium. This makes it likely that bacitracin has its natural function during growth, and not after growth as suggested by *Weinberg* (25).

The present comparison of the bacitracin producer and the non-producer mutant indicate that bacitracin is able to participate in the transport of several trace metals during growth of *B. licheniformis*. The metal chelator EDTA markedly inhibited the bacitracin-negative mutant whereas the bacitracin producer grew well. Adding excess Mn(II) ions, Co(II) ions, Zn(II) ions, or bacitracin to the EDTA inhibited mutant markedly promoted growth. This indicates that the lack of bacitracin production makes the mutant, *SB 319* less efficient in extracting trace metals from the environment. This suggestion is supported by the observation that a revertant of the non-producer with a restored bacitracin production, was not inhibited by the addition of EDTA. Inactivated bacitracin did not promote growth of the EDTA inhibited non-producer, indicating that the growth promotion observed by the addition of bacitracin was not due to impurities in the bacitracin solution used.

In a recent paper we have suggested that a possible function of bacitracin during growth of *B. licheniformis* may be to participate in the manganese(II) ion transport of the producer strain (13). Bacitracin is able to both bind to the cytoplasmic membrane (16) and to complex with Mn(II) ions (8). Bacitracin may therefore increase the uptake of Mn(II) ions by promoting the interaction between the transport mechanism for Mn(II) ions in the cytoplasmic membrane and the cation (13). Since bacitracin is able to also complex with Co(II) ions and Zn(II) ions (8), it is possible that bacitracin may promote the uptake of these cations in a similar manner.

The presence of trace metals is necessary

for the function of microbial cells. *Wyatt* (27) has pointed out that more than 100 enzymes are known to require metal ions such as Mn(II) Co(II) and Zn(II) ions as cofactors. Other enzymes may be inhibited by the same metal ions. *Wyatt* (27) suggests that these enzymes may be regulated by the relative concentrations of metal ions and ligands which bind the metal ions influencing the enzyme activity. *Eisenstadt et al.* (5) have suggested that all bacterial cells may have highly specific active transport systems for each cation which plays a critical metabolic role. The transport system for trace metals in *B. licheniformis* seems to be somewhat less specific since bacitracin is able to complex with several metal ions. It is possible that bacitracin is a common "helper molecule" for the different transport systems in the following manner: (i) bacitracin is excreted into the medium and complexes with trace metals present (ii) the bacitracin-metal-complexes bind to the cytoplasmic membrane (iii) the metal ion is transferred to its specific transport mechanism located in the membrane.

The *Bacillaceae* produces an array of peptide antibiotics with excellent metal binding properties. Possible functions of these chelating molecules may be to participate in the transport of metal ions, as may be the case with bacitracin, or they may participate in the control of enzyme activities by acting as ligands in the metal-ligand model described by *Wyatt* (27)

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REFERENCES

1. *Bernlohr R. W & Novelli, G D* Some characteristics of bacitracin production by *Bacillus licheniformis*. Arch. Biochem. Biophys. *87*: 232–238, 1960.
2. *Bernlohr R. W & Novelli, G D.*: Bacitracin biosynthesis and spore formation: the physiological role of an antibiotic. Arch. Biochem. Biophys. *103*: 94–104 1963
3. *Bodanszky M & Perlman, D*: Peptide antibiotics. Science, N Y *163* 352–358, 1969
4. *Demain A. L.*, How do antibiotic producing microorganisms avoid suicide? Annal. N Y Acad. Sci. *235* 601–612, 1974.
5. *Eisenstadt E., Fisher S., Der C L. & Silver S* Manganese transport in *Bacillus subtilis W23* during growth and sporulation. J Bact. *113* 1363–1372, 1973.
6. *Frøyshov O* Bacitracin biosynthesis by three complementary fractions from *Bacillus licheniformis*. FEBS Letters *44* 75–78, 1974
7. *Frøyshov O & Laland S G.*, On the biosynthesis of bacitracin by a soluble enzyme complex from *Bacillus licheniformis*. Eur J Biochem. *46* 235–242, 1974
8. *Garbutt J T., Morehouse A. L. & Hanson, A M.*, Metal binding properties of bacitracin. J Agr Food Chem. *9* 285–289 1961
9. *Hills G M Belton F C & Blatchley E. D.*, Ayfivin: production in chemically defined media and comparison with licheniformin. Brit. J Exp. Pathol. *30* 427–443 1949
10. *Hurst A.*, Biosynthesis of polypeptide antibiotics. In: *Gould G W & Hurst A.* (Ed.): The bacterial spore, (1.ed.) Academic Press, London and New York 1969 p. 167–182.
11. *Haavik, H I*: Studies on the formation of bacitracin by *Bacillus licheniformis* effect of glucose. J gen. Microbiol. *81* 383–390, 1974
12. *Haavik, H I & Thomassen S.*, A bacitracin-negative mutant of *Bacillus licheniformis* which is able to sporulate. J gen Microbiol. *76* 451 454 1973
13. *Haavik H I & Frøyshov O* Function of peptide antibiotics in producer organisms. Nature *254* 79–82 1975
14. *Ishihara, H., Sasaki T & Shimura, K.* Biosynthesis of bacitracin. II. Incorporation of [14]C-labelled amino acids into bacitracin by a cell-free preparation from *Bacillus licheniformis*. Biochim. Biophys. Acta *166* 496–504 1968.
15. *Kambe M Imae Y & Kurahashi K.* Biochemical studies on gramicidin S non-producing mutants of *Bacillus brevis ATCC 9999* J Biochem. *75* 481–493 1974
16. *MacDonald R. I., MacDonald R. C & Cornell, N W.*, Perturbation of liposomal and planar lipid bilayer membranes by bacitracin-cation complex. Biochem. *13*: 4018–4024 1974
17. *Maier W & Gröger D.*, Biosynthese der Peptidantibiotica. Die Pharmazie. *8*: 491–505 1972.
18. *Marschke C K & Bernlohr R. W.*, Reevaluation of bacitracin as a spore coat component. J Bact. *102* 283–284 1970.

19 *Ray B & Bose S K*: Polypeptide antibiotic negative sporeformer mutants of *Bacillus subtilis*. J Gen. Appl. Microbiol. *17*: 491–498, 1971
20. *Sadoff H L.* Sporulation antibiotics of *Bacillus* species. In *Halvorsen H C., Hanson R. & Campbell L. L.* (Ed.): Spores, vol. 5. American Society for Microbiology Washington 1972 p. 157–166
21 *Schaeffer P* Sporulation and the production of antibiotics, exoenzymes, and exotoxins. Bact. Rev *33*: 48–71 1969
22 *Schazschneider B., Ristow H & Kleinkauf H.*. Interaction between the antibiotic tyrocidine and DNA in vitro. Nature *249*: 757–759 1974
23 *Sarkar N & Paulus H.*: Function of peptide antibiotics in sporulation. Nature N.B. *239*: 228–230 1972.
24 *Snoke J E.* Amino acid composition of *Bacillus licheniformis* spore coat. Biochem. Biophys. Res. Comm. *6* 371 374 1964.
25 *Weinberg, E. D.*: Bacitracin, gramicidin, and tyrocidine. In *Gottlieb D & Shaw, P D* (Ed.) Antibiotics, 1.ed. vol. 2. Springer Verlag, Heidelberg and New York 1967 p. 240–253.
26. *Weinberg, E. D & Tonnis, S M.*: Action of chloramphenicol and its isomers on secondary biosynthetic processes of *Bacillus*. Appl. Microbiol. *14* 850–856, 1966.
27 *Wyatt H V.*. Cations, enzymes and control of cell metabolism. J Theoret. Biol. *6* 441–470, 1964

RAPID IDENTIFICATION OF GRAM NEGATIVE RODS USING A THREE TUBE METHOD COMBINED WITH A DICHOTOMIC KEY

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Lassen, J Rapid identification of Gram-negative rods using a three-tube method combined with a dichotomic key Acta path. microbiol. scand. Sect. B, 83 525–533 1975

A three-tube method combined with a dichotomic key is presented which will identify virtually all *Enterobacteriaceae* as well as a number of other frequently encountered Gram-negative rods at a genus or species level within 20 hours following the primary isolation. The method is shown to be reliable and simple, saving processing time and material.

Key words Gram-negative rods rapid identification; three-tube method dichotomic key

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A number of methods for the rapid identification of Gram-negative rods, especially rods belonging to the family *Enterobacteriaceae* have been introduced (1 5 6, 9 1-16) This reflects an obvious need in bacteriological laboratories concerned with routine diagnostic work.

Many of the methods are presented as commercially available systems and may have certain drawbacks The procedures are usually relatively expensive and may also be difficult to control. The continuous supply of media from the manufacturer may in some situations prove problematic, especially if delivered from a foreign country

This article presents a simple three tube method and a dichotomic key which will easily identify the *Enterobacteriaceae* and other frequently encountered Gram-negative rods at the level of the genus or species.

The media used may easily be prepared in any microbiological laboratory

MATERIAL AND METHODS

Cultures tested 453 cultures of Gram-negative rods, partly selected at random from various kinds of clinical specimens (418 cultures) and partly taken from the culture collection of the National Institute of Public Health, Oslo (35 cultures) were investigated

Media and reagents The three media used are described by Le Minor (10) and slightly modified by us.

I Combined lactose-glucose-H_2S-tube
(Hajna-tube")

Peptone (tryptic digest of fresh meat) (Merck) 20 g, lactose 10 g, glucose 1 g, sodium thiosul-

LACTOSE

H2S

INDOL · MOTILITY · MANNITOL · OXIDASE · UREA · GAS · GLUCOSE · MOTILITY 37° C · MOTILITY 22° C · O/W

Ent. cloacae LDC · Kleb. pneum. LDC · Ser. rubidea LDC · Ent. aerog. LDC · Ser. liquef. · Ser. rubid. · Pless. shigel. · Aer. hydr. · Klebs. ozaenae · Prot. vulg. · (Past. pneum.) · Citrob. freund. · Acinetobac. · Prot. m · Bord. bronch. · Salmonella sp. LDC · Arizona LDC ONPG · Citrob. freund. LDC · Edw. tarda · Flavob. sp. · (Past. mult.) · Alc. faec. · S. typhi · Salmonella sp. · (Past. haem.)

Prot. · Klebs. pneum. · Pseudomonas sp. · Alcaligenes · Shigella sp. · Prov. alc. · Enterobacter sp. ONPG · Serratia sp. ONPG · Salmonella sp. ONPG · Ent. haf. · Flavobact. sp. · Acinet. · Klebs. ozaenae · Ser. marc. · Yer. pseud. · Yer. enterc. · Vibr. cholerae · TDA · LDC

Possibilities marked with * ... occasionally ...

Fig. 1 Key 1 Identification of Gram-negative rods

LACTOSE

H_2S

INDOL

INDOL

UREA

MOTILITY

*E. ... Citr. ... LDC

Arizona ... sp. LDC

Ent. cloacae LDC

Ser. lique ... LDC /

*Ser. rubid. LDC /

Kl. b. pneum. LDC

*Kleb. ... LDC d

UREA

GAS

GAS

MOTILITY

*Y. ... ent.

Kleb. pneum.

E. col

MOTILITY

E. col

*Dispar LDC ... Sh. boyd. 9 LDC

MOTILITY

GAS

Ent. aerog LDC

Ent. ... LDC

*Ser. ... LDC /

Ser. ... S. ... liq.

Kl. b. pneum.

Kl. b. ozaenae

UREA

MANNITOL

Cit. ...

INDOL

Prot. vulg. Pr. ...

INDOL

MANNITOL

Salmonell sp. LDC

Citrob. ... LDC

Edwardsiella

GAS

Salmon ... sp. LDC ONPG

S. typhi

Arizona ... LDC ONPG /

Citrob. ... LDC ONPG

UREA

INDOL

INDOL

MANNITOL

MOTILITY

Prot. morg.

GAS

Yers. ent.

MANNITOL

*Prot. mirabilis

MOTILITY 37°C

MOTILITY 22°C

Yers. ent.

Yers. pseudotub.

Kleb. ozaenae LDC d

Kleb. p. ... LDC

GAS

Ent. cloacae LDC

Ser. lique ... LDC /

Ser. marc. ...

MANNITOL

GAS

E. coli

MOTILITY

Alcale... LDC + /

Shigella sp. LDC

Pr. ... stuartii TDA

Pre. stuarti ... TDA

E. c ... TDA

GAS

Pr. ... alc.

MOTILITY

Shigell sp. TDA

Prov. stuartii TDA +

Providencia sp.

MANNITOL

GAS

MOTILITY 37°C

Enterobacter sp. ONPG

Serr. ... sp. ONPG

Salmonella sp. ONPG

MOTILITY 22°C

E. ... hafnia

Ser. lique ...

Kleb. ozaenae

MOTILITY

S. typhi

*Salmonella sp. ONPG

Ser. marcesc. ONPG

Shigella sp.

Kleb. ozaenae

Kleb. rhinosc...

Shigella sp.

* ... mark ... d with ... r ... ely occurring

Fig 2 Key 2 Identification of *Enter bacteriaceae*

TABLE 1 *Summary of the Biochem*

Bacterial group / Test or substrate§	LAC	GLU	GAS	H_2S	MAN	MOT_{37}	MO
Escherichia coli	+/—	+	+/—	—/+	+	+/—	+/
"Alcalescens"	—	+	—	—	+	—	—
"Dispar"	+	+	—	—	+	—	—
Shigella sp.	—/+	+	—/+	—	d	—	—
Edwardsiella tarda	—	+	+	+	—	+	+
Enterobacter aerogenes	+/—	+	+	—	+	+	+
E. cloacae	+/—	+	+	—	+	+	+
E. hafniae	—/(+)	+	+	—	+	+/—	+
Klebsiella pneumoniae	+/—	+	+	—	+	—	—
K. ozaenae	d	+	d	—	+	—	—
K. rhinoscleromatis	(d)	+	—	—	+	—	—
Serratia marcescens	—	+	— or w	—	+	+	+
S. liquefaciens	d	+	d	—	+	d	+
S. rubidaea	+	+	— or w	—	+	+	+
Salmonella sp.	—	+	+/—	+/—	+	+	+
S. typhi	—	+	—	w	+	+	+
Arizona hinshawii	d	+	+	+	+	+	+
Citrobacter freundii	d	+	+	+	+	+	+
C. diversus	d	+	+	—	+	+	+
Proteus vulgaris	—	+	+/—	+	—	+	+
P. mirabilis	—	+	+	+	—	+	+
P. morganii	—	+	d		—	d	d
P. rettgeri	—	+	d	—	+/—	+	+
Providencia stuartii	—	+	—	—	d	d	d
P. alcalifaciens	—	+	d		—	+	+
Yersinia enterocolitica	—/+	+	—	—	+	—	+
Y. pseudotuberculosis	—	+	—	—	+	—	+
Pasteurella multocida	—	+	—	d	+	—	—
P. pneumotropica	d	+	—	d	—	—	—
P. haemolytica	d	+	—	d	+	—	—
P. ureae	—	+	—	—	+	—	—
Actinobacillus lignieresii	(+)	+	—	+	+	—	—
Flavobacterium sp.†	—/(+)	d	—	— or w	(d)	—	—
Alcaligenes faecalis	—	—	—	d	—	+	+
Bordetella par. pertussis	—	—	—	—	—	—	—
B. bronchiseptica	—	—	—	d	—	+	+
Acinetobacter calcoaceticus†	d	d	—		d	—	—
Moraxella sp.	—	—	—	—	—	—	—
Vibrio cholerae	(+)	+	—	—	+/(+)	+	+
Aeromonas hydrophila	d	+	d	d	+	+	+
Plesiomonas shigelloides	(+)	+	—	—	—	+	+
Pseudomonas sp.†	—	+	—	—	d	+	+

According to Bergey's Manual of Determinative Bacteriology (2), *Cowan & Steel* (4), *Edwards &*
Ewing (5) and *Ewing* (7)

§ LAC: Lactose, GLU: Glucose, GAS: Gas from glucose, MAN: Mannitol, MOT_{37}: Motility at 37°C,
MOT_{22}: Motility at 22°C, IND: Indol, NIT: Nitrate reduction, OXI: Oxidase, LDC: Lysinede-
carboxylase, ONPG: Beta-galactosidase, TDA: Tryptophane- or phenylalaninedeaminase, ADH: Argi-
ninedihydrolase, INO: Inositol, LIP: Lipase corn oil, VP: Voges-Proskauer, MAL: Malonate, ARA:
Arabinose, DUL: Dulcitol, ODC: Ornithinedecarboxylase, *SAC*: Saccharose.

…ions of the Gram-negative Rods

IA	IND	NIT	OXI	LDC	ONPG	TDA	Additional tests
—	+	+	—	d	+	—	
—	+	+	—	d	—	—	
—	+	+	—	d	+	—	
—	d	+	—	—	d	—	
—	+	+	—	+	—	—	
—	—	+	—	+	+	—	ADH — INO + LIP —
d	—	+	—	—	+	—	ADH + INO d, LIP —
—	—	+	—	+	+	—	ADH — INO — LIP —
+	—/+	+	—	+	+	—	VP + MAL +
d	—	+	—	d	+	—	VP: — MAL —
—	—	+	—	—	+	—	VP — MAL +
— w	—	+	—	+	+	—	LIP: + ARA: — ODC +
d	—	+	—	d	+	—	LIP + ARA: + ODC +
d	—	+	—	d	+	—	LIP + ARA + ODC —
—	—/+	+	—	+	—	—	MAL — DUL: +
—	—	+	—	+	—	—	
—	—	+	—	+	+/—	—	MAL: + DUL —
d	—	+	—	—	+	—	
+	+	+	—	—	+	—	
+	+	+	—	—	—	+	ODC — INO — LIP d
+	—	+	—	—	—	+	ODC: + INO — LIP +
+	+	+	—	—	—	+	ODC: + INO — LIP —
+	+	+	—	—	—	+	ODC: — INO + LIP —
—	+	+	—	—	—	+	INO +
—	+	+	—	—	—	+	INO —
+	d	+	—	—	+	—	SAC: + ODC +
+	—	+	—	—	+	—	SAC — ODC: —
—	+	+	d	—	d		
+	+	+	+	—	+		
—	—	+	+	—	d		
+	—	+	+	—	—		
+	—	+	d		+		
—	d	—	+				
—	—	d	+				
	—	—	—				Growth at 42 C —
+	—	+	+				
d	—	—	—	—			Growth at 42 C +
—	—	d	+				
—	+	+	+	+	+		LIP +
—	+	+	+	—	+		
—	+	+	+	+	+		LIP —
d	—	d	+	—/+			

KEY + 90–100 per cent strains are positive, d 10–90 per cent strains are positive —: 0–10 per cent strains are positive. (+) Delayed reaction, w: Positive, but weak reaction, +/— Majority of strains positive, few strains negative, —/+: Majority of strains negative, a few strains positive. (d) Different reactions, positives delayed.

† Attack sugars by oxidation.

phate ($Na_2S_2O_3$ 5 H_2O) 0.2 g, ferrous ammonium sulphate ($(NH_4)_2Fe(SO_4)_2$ 6 H_2O) 0.3 g, sodium chloride (NaCl) 5 g, agar 17 g phenol red 0.2 per cent 12.5 ml, distilled water ad 1000 ml.

pH is adjusted to 7.0 The substrate is heated in a boiling water bath and distributed in tubes (15-16 × 150 mm) with screwcaps, 6.5 ml in each. After autoclaving 15 minutes at 121 C, the tubes are placed at an angle of approximately 5 C to form a slope beginning about 4 cm from the bottom.

The medium demonstrates the following reactions

1 Fermentation of glucose yellow butt. Gas formation raises the medium from the bottom of the tube and/or breaks it up.
2 Fermentation of lactose yellow slope (The *screw-cap must not be closed too tightly for this* reaction to occur)
3 H_2S-production blackening of the medium.
4 Lysine decarboxylase: Following overnight incubation, 4 ml 4N sodium hydroxide (NaOH) and 2 ml chloroform are added to the medium. A clear chloroform phase covered by a cloudy fluid is obtained, the latter is removed with a Pasteur pipette and discarded. By another pipette approximately 1 ml of the chloroform phase is transferred to a smaller tube, discarding the first and the last drops of fluid. An equal amount of 0 1 per cent ninhydrine in chloroform is added and the tube left in an upright position for 10 minutes. A violet colour indicates a positive reaction.

Material from the slope may also be used to carry out tests for beta-galactosidase, phenylalanine desminase and oxidase (10)

II. Mannitol-motility-tube

Peptone (tryptic digest of casein) (Merck) 5 g, Neopeptone (Difco) 5 g mannitol 2 g, agar 2.5 g potassium nitrate (KNO_3) 1 7 g phenol red 0.2 per cent 20 ml, distilled water ad 1000 ml

pH is adjusted to 7 4 The substrate is heated in a boiling water bath and distributed in tubes (15-16 × 150 mm) 5 ml in each, and autoclaved for 15 minutes at 121 C.

The medium demonstrates the following reactions

1 Fermentation of mannitol yellow colour
2. Motility the growth of non-motile microbes will be restricted to the central stab line. Motile microbes will spread throughout the medium and make it turbid.
3 Nitrate reduction after overnight incubation 4 drops of each of the following solutions are added to the medium A. Sulphanilic acid (1 g in 100 ml 5N acetic acid) B. Dimethyl-alpha naphtylamine (0.6 ml in 100 ml 5N acetic acid) The presence of nitrite is indicated by a red colour A negative nitrate-reduction test can be confirmed by adding a minute amount of zinc dust to the medium. The presence of unreduced nitrate is indicated by the development of a red colour

III Urea-indol-tube

L-tryptophane 0.3 g, potassium dihydrogen phosphate (KH_2PO_4) 0.1 g, dipotassium hydrogen phosphate (K_2HPO_4) 0.1 g, sodium chloride (NaCl) 0.5 g, urea (H_2NCONH_2) 2.0 g, ethanol 96 per cent 1 ml, phenol red 0.2 per cent 1.25 ml, distilled water ad 100 ml.

pH is adjusted to 6.5. The solution is filter sterilized (pore size 0.22 µm) and distributed in small tubes (10-11 × 120 mm) 0.6 ml in each.

The medium demonstrates the following reactions

1 *Urease positive reaction* is indicated by a red colour of the medium
2. Indol production after overnight incubation, 0.5 ml of Kovacs' reagent (8) is added to the medium. The tube is gently shaken. The presence of indol is indicated by a pink to deep red colour in the upper layer of the fluid.
3 Tryptophane deaminase after overnight incubation and before adding Kovacs reagent, four drops of the medium are transferred to a small tube. One drop 10 per cent ferric chloride ($FeCl_3$) is added. Positive reaction is indicated by the development of a reddish-brown colour

Inoculation and incubation A single colony of the Gram-negative rods is suspended in the urea-indol-medium. The other two media are inoculated from this suspension by an ordinary bacteriological loop the mannitol-motility-medium by stabbing the loop into the centre of the medium, the "Hajna"-medium by first smearing the slope and then stabbing the loop into the lower part of the medium along the wall to the bottom of the tube. The suspension is finally plated on a suitable agar (e.g. MacConkey) to check the purity of the suspended colony

If a single colony is too small to make the suspension in the urea-indol-medium visibly turbid, this medium may be incubated at 37° C for 3-4 hours before the other two media are inoculated When starting from a pure culture, several colonies may be simultaneously suspended.

Immediately after the inoculation, the tubes are placed in an incubator at 37° C to avoid possible motility at room temperature. If it is necessary to examine for motility at 22 C, an additional mannitol-motility medium has to be inoculated and incubated at this temperature.

The tubes are incubated for 18-22 hours.

The dichotomic keys Two keys are presented; one concerning the identification of Gram-negative

ods, including *Enterobacteriaceae* (Key 1 Fig. 1) and one which is restricted to the identification of *Enterobacteriaceae* (Key 2, Fig 2)

The keys are based on the reactions that may be read directly from the three media after the prescribed incubation. In addition, the oxidase reaction (Kovacs' method) is used in Key 1

Additional tests which can be carried out in few minutes using material from the three media (lysine decarboxylase, beta-galactosidase, nitrate reduction, phenylalanine deaminase) are only cited if it is necessary to distinguish between bacterial groups placed together in the keys.

The traditional classification of *Enterobacteriaceae* as lactose-negative or -positive is taken into account, lactosefermentation being used as the first (the superior) criterion. This is followed by criteria which are regarded as relatively stable within the bacterial groups, and finally by those which are regarded as less stable (gas production and motility)

Most of the bacterial groups are listed in more than one place in the keys, reflecting their variable reactions to one or more of the tests.

The nomenclature for *Enterobacteriaceae* is based on the proposals made by *Edwards & Ewing* (5) and *Ewing* (7) the nomenclature for the other groups of bacteria is based on Bergey's Manual of Determinative Bacteriology (2)

Most of the identifications made are achieved on the basis of less than the total number of reactions given by the three media. Table 1 shows a summary of the reaction patterns with the given tests for all the bacterial groups cited in the keys, according to Bergey's Manual of Determinative Bacteriology (2) *Cowan & Steel* (4) *Edwards & Ewing* (5) and *Ewing* (7)

Identification. All the investigated strains were finally identified according to *Edwards & Ewing* (5) *Ewing* (7) and *Cowan & Steel* (4) using conventional media and reagents (8, 10)

The identifications of Salmonella sp., Shigella sp. and Yersinia sp. were also serologically verified.

Lysine d carboxylase test The lysine decarboxylase test, carried out as described under the "Hajna"-medium, was compared with the test carried out with Carlquist s medium and with the modified Falkow' medium (10) by testing 84 randomly selected strains by all three methods.

RESULTS

The identifications of the investigated strains achieved by the three-tube method are compared with the final identifications achieved by the conventional system in Table 2.

TABLE 2. *Identification of 453 Strains of Gram egative Rods by the Thr e-tube Method Compared with Conventional Identification*

Organism	C/T	Organism	C/T*
Escherichia coli	20/23**	*Proteus vulgaris*	4/4
Alcalescens"	3/3	*Proteus mirabilis*	13/13
"Dispar"	2/2	*Proteus morganii*	11/11
Shigella sp.	62/62	*Proteus rettgeri*	7/7
Shigella boydii 9	1/1	Providencia sp.	2/2
Edwardsiella tarda	1/1	*Providencia stuartii*	2/2
Enterobacter sp.	17/17	*Providencia alcalifaciens*	2/2
Enterobacter aerogenes	4/5**	Pseudomonas sp.	11/11
Enterobacter cloacae	2/2	*Aeromonas hydrophila*	4/4
Enterobacter hafniae	11/11	*Yersinia enterocolitica*	80/80
Klebsiella pneumoniae	11/13**	*Yersinia pseudot berculosis*	8/8
Klebsiell ozaenae	3/3	*Acinetobacter calcoaceticus*	15/15
Klebsiella rhinoscleromatis	2/?	*Alcaligenes faecalis*	5/5
Serratia mar escens	7/7	*Bordetella bronchiseptica*	1/1
Serratia liquefaciens	2/2	*Vibrio cholerae*	4/4
Salmonella sp.	100/100	Moraxella sp.	1/1
Salmonella typhi	4/4	Unidentified	0/2**
Arizona hinshawii	3/3		
Citrobacter freundii	?0/?0	TOTAL	445/453

C/T: No. correct per no. tested.
** The identification errors are explained under Results

The overall agreement between the two systems is 98.2 per cent (445/453)

Three indol-negative strains of *E. coli* were classified by the three-tube method as Enterobacter sp., *Enterobacter aerogenes* and *Enterobacter cloacae* respectively

Two urease-negative strains of *Klebsiella pneumoniae* were classified as *Klebsiella ozaenae*

One strain of *Enterobacter aerogenes* which was non-motile at 37 C, was classified as *Enterobacter hafniae*

Two strains which were classified as *Aeromonas hydrophila* and *Alcaligenes faecalis* respectively could not be finally identified.

There was complete agreement between the three methods used for the lysine decarboxylase test. Of the 84 tested strains, 37 showed a positive reaction and 47 a negative reaction

DISCUSSION

Using the three-tube method and the dichotomic keys, virtually all *Enterobacteriaceae* can be identified at a genus- or species level within 20 hours following the primary isolation. The method will also identify a number of other Gram-negative rods (e.g. Pseudomonas sp *Aeromonas hydrophila Alcaligenes faecalis Vibrio cholerae*)

Some other Gram negative rods do not grow sufficiently well on these media to give reliable results (e.g. most of the species belonging to the genus *Pasteurella*) The same tests carried out with adequate media and reagents may however be used for the primary classification also for a number of such organisms, and they are therefore placed in brackets in Key 1

Reaction patterns occurring as rare exceptions have been excluded from the keys and may therefore lead to identification errors. Thus indol negative *E coli* strains (approximately 1.5 per cent of the *E coli* strains (5)) will be identified as Enterobacter indol-negative *Proteus vulgaris* strains (approximately 1.8 per cent (5)) as *Proteus mirabilis* and indol-positive *Proteus mirabilis* strains (approximately 1.9 per cent (5)) as *Proteus vulgaris* Non motile Enterobacter sp. may be identified as Klebsiella sp.

The biochemical differentiation between species belonging to Serratia and species belonging to Enterobacter may sometimes need supplementary tests (see Table 1) The majority of the isolated Serratia strains may however be recognised by their pigmentproduction.

The biochemical differentiation of the species *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* also needs supplementary tests (see Table 1) They must nonetheless be serologically verified.

Some Shigella species will show the same reaction pattern as *Klebsiella rhinoscleromatis* and some lactose-negative *Klebsiella ozaenae* strains These genera can, however be separated on the basis of the mucoid colonies of the Klebsiella species and the Shigella species will also have to be serologically verified.

Alcaligenes faecalis and Pseudomonas sp. sometimes show identical reaction patterns, but may be separated by different colony morphology and by the pigment and odour produced by the majority of the isolated Pseudomonas strains.

The lysine decarboxylase test, carried out on material from the "Hajna medium, has been shown to have the same sensitivity and specificity as that obtained if Carlquist's medium or the modified Falkow's medium are used.

The results of the tryptophane deaminase test, carried out on material from the urea-test, indol-medium may occasionally be difficult to interpret. This test therefore appears less suitable than the conventional phenylalanine deaminase test.

The described method, using polytropic media, is a simple and rapid method for the routine identification of the most commonly isolated Gram-negative rods. In principle, all polytropic media will, however provide a lower degree of accuracy than monotropic media and should not be used in reference or scientific work.

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REFERENCES

1. *Borchardt K. A.* Scheme for screening and identifying enteric and other Gram-negative bacteria using reagent-impregnated strips. Amer. J. Clin. Path. *50* 129–131 1968.
2. *Buchanan, R. E. & Gibbons N. E.* Bergey's Manual of Determinative Bacteriology. 8. ed. Williams & Wilkins Company Baltimore 1974.
3. *Clase O. & Digranes, A.* Rapid identification of prompt lactose-fermenting genera within the family *Enterobacteriaceae*. Acta path. microbiol. scand. Sect. B *79* 673–678, 1971
4. *Cowan S. T. & Steel, K. J.* Manual for the identification of medical bacteria. 2. ed. Cambridge University Press, Cambridge 1974
5. *Edwards P. R. & Ewing, W. H.* Identification of *Enterobacteriaceae*. 3. ed. The Burgess Publishing Company Minneapolis 1972
6. *Elston H. R., Baudo J. A., Stanek, J. P. & Schaab M.* Multibiochemical test system for distinguishing enteric and other Gram-negative bacilli. Appl. Microbiol. *22* 408–414 1971
7. *Ewing, W. H.:* Differentiation of *Enterobacteriaceae* by biochemical reactions. Revised. Center for Disease Control, Atlanta 1973.
8. *Ewing, W. H. & Davis B. R.:* Media and tests for differentiation of *Enterobacteriaceae* Center for Disease Control Atlanta 1970
9. *Johnstone G. F. & Brown, S. E.* A short scheme for the rapid identification of Gram-negative bacilli in urine. J. med. Lab. Technol. *27* 222–228, 1970
10. *Le Minor L.* Le diagnostique de Laboratoire des *Entérobactéries*. 3. ed. De la Tourelle, St. Mandé, 1967
11. *Rhoden, D. L. Tomfohrde K. M., Smith, P. B. & Balows A.* Auxotab—a device for identifying enteric bacteria. Appl. Microbiol. *25* 284–286, 1973
12. *Roberts G.* The rapid identification of Gram-negative bacilli. Med. Lab. Technol. *28* 382–384 1971
13. *Rosner R. F.* Identification of enteric bacilli directly from primary isolation media using reagent-impregnated strips. Amer. J. Clin. Path. *54* 587–595 1970.
14. *Smith, P. B. Rhoden D. L., Tomfohrde K. M. Dunn, C. R. & Hermann, G. J.* R/B enteric differential system for identification of *Enterobacteriaceae* Appl. Microbiol. *21:* 1036–1039 1971
15. *Smith, P. B. Tomfohrde K. M., Rhoden D. L. & Balows A.* API system, a multitube micro method for identification of *Enterobacteriaceae*. Appl. Microbiol. *24* 449–45., 1972.
16. *Tomfohrde K. M., Rhoden D. L. Smith, P. B. & Balows A.* Evaluation of the redesigned Enterotube—a system for the identification of *Enterobacteriaceae* Appl. Microbiol. *25* 301–304 1973.

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BACITRACIN PRODUCTION BY THE NEOTYPE

Bacillus licheniformis ATCC *14580*

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Haavik, H. I Bacitracin production by the neotype *Bacillus licheniformis* ATCC *14580* Acta path. microbiol. scand. Sect. B, 83 534–540 1975

A small but significant bacitracin production has been observed in the neotype *Bacillus licheniformis* ATCC *14580* This strain produces bacitracin only during the phase of rapid growth. The bacitracin production ceases before growth is completed, and the relationship between growth and antibiotic production was not influenced by different environmental conditions. The maximum titre produced by the neotype strain was about 1 i.u. bacitracin/ml. This maximum titre was not influenced by the environmental conditions which resulted in a great variability of the maximum titre of the known bacitracin producer *Bacillus licheniformis* ATCC *10716* In contrast to *B. licheniformis* ATCC *10716* the bacitracin production of the neotype *B. licheniformis* ATCC *14580* was not stimulated by Mn(II)ions. It is suggested that bacitracin may be incidentally overproduced by certain strains grown in certain environments. The controlled bacitracin production by the neotype is not consistent with the definition of secondary metabolites and the hypothesis concerning the function of secondary metabolites after growth of the producer organisms.

Key words Bacitracin production *Bacillus licheniformis* ATCC *14580*

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The production of the peptide antibiotic bacitracin has been extensively studied mainly in two strains *Bacillus licheniformis* strains A 5 and ATCC *10716* (2 3 9 10 16) Both strains produce relatively large amounts of the antibiotic with a maximum titre of about 50 i.u. bacitracin/ml. It is suggested that the abundant production of secondary metabolites (such as peptide antibiotics) serves a control function (4) or a detoxification function (19) during the transition from active growth to stationary phase metabolism. Recently however *Sadoff* (15) has suggested that the known antibiotic producers are incidentally overproducing their antibiotics due to a defect control mechanism. The normal event is to produce very small amounts of the peptide antibiotics and *Sadoff* (15) suggests that these peptide antibiotics may control the sporulation process of the aerobic bacilli.

We have recently described a bacitracin-negative mutant which sporulates normally (8, 12) It is therefore not likely that bacitracin serves a control function during the sporulation process of *Bacillus licheniformis*.

The aim of the present work was to investigate the neotype *Bacillus licheniformis* ATCC *14580* for bacitracin production and to compare it with that of *B. licheniformis* ATCC *10716*.

MATERIALS AND METHODS

Reagents

All reagents used were of analytical grade. The bacitracin used has been described previously (13).

Media and Growth Conditions

The chemically defined media (M12 and M20) for growth and bacitracin production by *Bacillus licheniformis* ATCC *10716* and ATCC *14580* and conditions for incubation have been described previously (9). The inoculum consisted of spores (9).

Growth

Bacterial growth was measured as extinction at 650 nm using a spectronic 20 spectrophotometer as described (10).

Assay of Bacitracin

The bacitracin produced was determined by an agar diffusion method described previously (8). The sensitivity of the method was improved by lowering the amount of test organism (*Micrococcus flavus*) in the agar medium.

RESULTS

Bacillus licheniformis ATCC *14580* produced small but significant amounts of bacitracin in the different media used. The antibiotic was identified as bacitracin by the thin-layer chromatography method described previously (8). Table 1 shows that bacitracin was produced during the active growth phase in the M12 medium. However the bacitracin production ceased before growth was completed. The amounts detected during later stages of growth gradually decreased.

The addition of 1 per cent glucose to the medium showed only minor effects upon the culture. Table 2 shows that bacitracin was only produced during the active growth phase and that the bacitracin production ceased before growth was completed. The bacteria showed a slightly more rapid growth and somewhat higher antibiotic titres when glucose was a constituent of the medium. In this medium the pH dropped to about 6.0 during early growth.

The buffer capacity of the medium was increased by increasing the phosphate content by a factor of ten and by lowering the initial pH to 6.0 (M20 medium). When *B. licheniformis* ATCC *10716* was grown in this M20 medium, the maximum titre was about 40 I.u. bacitracin/ml which was a marked increased production as compared with the 12 I.u. bacitracin/ml produced in the M12 medium (9, 10). Table 3 shows that *B. licheniformis* ATCC *14580* produced same amounts of bacitracin in this M20

TABLE 1. *Growth and Bacitracin Production by the Neotype* Bacillus licheniformis *ATCC* 14580 *in the M2 Medium*

Time of incubation (h)	Growth (E_{650})	pH	Bacitracin production (I.u./ml)
8	0.14	7.38	0.04
10	0.40	7.60	0.10
12	0.84	8.02	0.32
14	2.38	8.30	0.60
16	4.90	8.48	0.96
18	5.40	8.52	0.66
20	6.55	8.58	0.48
24	8.50	8.63	0.18
28	8.40	8.70	0.15

TABLE 2. *Growth and Bacitracin Production by the Neotype* Bacillus licheniformis *ATCC* 14580 *in the M2 Medium with 1 Per Cent Glucose*

Time of incubation (h)	Growth (E_{650})	pH	Bacitracin production (I.u./ml)
8	0.34	6.83	0.10
10	1.34	6.05	0.42
12	3.04	6.15	0.84
14	5.10	7.12	1.22
16	6.20	7.48	0.80
18	7.50	7.66	0.58
20	8.40	7.9?	0.30
24	9.30	8.45	0.14
28	9.00	8.56	0.10

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BACITRACIN PRODUCTION BY THE NEOTYPE

Bacillus licheniformis ATCC *14580*

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We have recently described a bacitracin-negative mutant which sporulates normally (8, 12) It is therefore not likely that bacitracin serves a control function during the sporulation process of *Bacillus licheniformis*.

TABLE 5. *Effect of Mn(II)Ions upon Growth and Bacitracin Production by* Bacillus licheniformis *ATCC 10716 (M2 Medium)*

Time of incubation (h)	Mn(II)Ions added (mg/l)					
	0.0		0.1		10.0	
	Growth (E_{600})	Bacitracin production (i.u./ml)	Growth (E_{600})	Bacitracin production (i.u./ml)	Growth (E_{600})	Bacitracin production (i.u./ml)
10	0.24	0.14	0.24	0.12	0.22	0.10
12	0.64	0.30	0.70	0.34	0.60	0.30
14	2.00	1.00	2.00	1 14	2.10	1.24
16	5.56	2.4	5.90	4 7	5.20	5.6
18	6.60	2.4	6.80	5.8	8.20	8.0
20	7 40	2.4	8.00	6.6	7.80	10.1
24	7.30	2.3	8.10	6.0	8.20	12.2
28	7.00	2.3	7 70	5.2	7.60	10.7

to be produced after growth of the producer strain *Bacillus licheniformis* A 5 (2, 3)

In contrast to this we have recently reported that bacitracin was produced after growth of *B. licheniformis* ATCC *10716* due to incidentally inhibitory effects of low pH which developed during early growth (9) The low pH arose from the rapid metabolism of glucose and the excretion of organic acids into the medium (11) Thus, in media without glucose, or when pH was controlled, bacitracin was produced by growing cells of *B licheniformis* ATCC *10716* (9 10 11)

Audhya & Russell (1) have reported that the antibiotic enniatin was produced after growth of *Fusarium sambucinum* only when the growth of the organism resulted in a drop in pH during early growth. When grown on a slowly metabolized carbohydrate, enniatin production paralleled growth. This supports our previous suggestion that peptide antibiotics may be produced after growth due to the development of unfavourable pH values during early growth (9 11)

In contrast to *B licheniformis* ATCC *10716* (9 10 11) the neotype *B. licheniformis* ATCC *14580* showed the same relationship between growth and antibiotic production at all environmental conditions examined. Small but significant amounts of bacitracin were produced only during the phase of active growth.

The bacitracin production of *B. licheniformis* ATCC *10716* and ATCC *14580* during active growth is not consistent with the general opinion that secondary metabolites are produced by cells that have recently stopped dividing (4 5 17)

The strain *B licheniformis* ATCC *10716* shows a considerable variation of the maximum titre, depending upon the cultural conditions (9 10 11) Thus the maximum titre of this strain may vary between about 2 and 50 i.u. bacitracin/ml. In contrast, the neotype *B. licheniformis* ATCC *14580* produces a maximum titre of about 1 i.u. bacitracin/ml at all cultural conditions used. From these data it is reasonable to assume that *B licheniformis* ATCC *10716* is overproducing baci-

TABLE 6. *Effect of Mn(II)Ions upon the Maximum Titre of Bacitracin Produced by* Bacillus licheniformis *ATCC 10716 Grown in the M20 Medium (Initial pH 6)*

Mn(II) Ions added (mg/l)	Growth after 26 h (E_{600})	Maximum titre of bacitracin (i.u./ml)
0 0	6.90	2.5
0.1	8.64	10.6
0.2	9.12	17 4
0.5	8.88	34 4
1 0	9 45	36.6
10 0	8.50	43 0

tracin due to a defect control mechanism as suggested by *Sadoff* (15) The neotype *B licheniformis* ATCC *14580* however probably exerts a strong control with its bacitracin formation and produces only the small amounts necessary for the function of bacitracin. We have previously suggested that bacitracin may act as a promotor between the cytoplasmic membrane and several trace metals present in the medium (12 13 14) Thus bacitracin may have a catalytical function in the trace metal transport and only small amounts will then be necessary It is reasonable to assume that the important trace metals are extracted rapidly from the medium during the first period of growth. Bacitracin production during later stages of growth may then not be necessary This may explain why bacitracin production by *B licheniformis* ATCC *14580* ceases before growth is completed.

The bacitracin production during later stages of growth of *B. licheniformis* ATCC *10716* was markedly stimulated by Mn(II) ions. The bacitracin production during early stages of growth, however was not significantly stimulated. At very low concentrations of Mn(II)ions the bacitracin production by *B. licheniformis* ATCC *10716* ceased before growth was completed, as was the case with the neotype *B licheniformis* ATCC *14580* at all cultural conditions. It is reported that the bacitracin synthetase the enzyme complex responsible for the synthesis of bacitracin, is markedly stimulated *in vitro* by Mn(II)ions (7)

The bacitracin production of *B licheniformis* ATCC *14580* was not stimulated by Mn(II)ions. It is possible that the neotype strain is controlling its bacitracin production by preventing an interaction between Mn(II)ions and the bacitracin synthetase. The growth of the neotype strain is markedly inhibited by high concentrations of Mn(II) ions. The bacitracin synthetase is therefore not prevented from interaction with Mn(II) ions due to lack of uptake of the ion. It is possible that the bacitracin synthetase is protected by means of chelating agents as in the enzyme-metalion ligand model described by *Wyatt* (20) *B licheniformis* ATCC *10716* may then be overproducing bacitracin due to lack of internal control with the Mn(II)ion concentration by means of sufficient amounts of ligands. This is supported by the observation that *B. licheniformis* ATCC *10716* is not overproducing bacitracin in environments low in Mn(II)ions.

It is reported that concentrations of inorganic phosphate in excess of that necessary for growth may distort the production of most secondary metabolites (18) Thus it is shown that excess phosphate reduced the maximum bacitracin titre by 83 per cent (18) Due to these observations, *Weinberg* (18) has suggested that inorganic phosphate may control the formation of secondary metabolites by suppressing phosphatases necessary for their formation.

During an investigation of the effect of phosphate upon bacitracin production by *B. licheniformis* ATCC *10716* it became apparent that excess phosphate indirectly affected the bacitracin titre by affecting the pH development of the culture (10) At very high concentrations of inorganic phosphate, a marked buffering action was introduced into the medium and very high titres of bacitracin were obtained (10) In contrast to *B licheniformis* ATCC *10716* the bacitracin production of *B. licheniformis* ATCC *14580* was not affected by excess inorganic phosphate. These observations are not consistent with the hypothesis that inorganic phosphate may control secondary metabolism. It seems as if inorganic phosphate only affects bacitracin production indirectly and only in strains which are overproducing the secondary metabolite.

The true function of secondary metabolites and secondary metabolism in the life cycle of the producing organism is widely unknown. The "Maintenance hypothesis" of *Bu Lock* (4) states that the function of secondary metabolites is to maintain the machinery essential to the cell division in operative order during phases when growth is no longer possible. When conditions for growth again

becomes favourable, the cells can almost immediately start dividing and thus have a selective advantage. The "Unbalanced Growth Hypothesis" of *Woodruff* (19) suggests that ordinary control mechanisms in certain organisms are not able to prevent the over synthesis of certain primary metabolites when conditions for cell division disappear. In order to avoid toxic concentrations inside the cells, the primary metabolites are converted into secondary metabolites which are excreted from the cells. The production of bacitracin only during the period of rapid growth of *B. licheniformis* ATCC *14580* is not consistent with these two hypotheses.

The definition of a secondary metabolite states that (i) they are produced after growth (ii) they have a very restricted taxonomic distribution (iii) they have no obvious function during growth of producer cells (4, 17). Our results show that bacitracin may be produced during growth and may also have a function during growth of the producer organisms. *Sadoff* (15) has suggested that most *Bacillus* species may produce bacitracin in relatively small amounts. Consequently the secondary metabolite bacitracin is no longer in agreement with the general definition of a secondary metabolite. In fact, the peptide antibiotic bacitracin seems to behave very similar to an ordinary primary metabolite which is incidentally overproduced in certain strains. Similarly it is possible that many secondary metabolites will turn out to be primary metabolites which are incidentally overproduced by certain strains in certain environments.

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REFERENCES

1. *Audhya, T. K. & Russell, D. W.*: Enniatin production by *F. sam. sambuci*: primary, secondary and unitary metabolism. J. gen. Microbiol. *86* 327–331 1975.
2. *Bernlohr R. W. & Novelli G. D.*: Some characteristics of bacitracin production by *Bacillus licheniformis*. Arch. Biochem. Biophys. *87* 232–238, 1960.
3. *Bernlohr, R. W. & Novelli, G. D.*: Bacitracin biosynthesis and spore formation: the physiological role of an antibiotic. Arch. Biochem. Biophys. *103* 94–104 1963.
4. *B. Lock, J. D.*: Intermediary metabolism and antibiotic synthesis. Advan. Appl. Microbiol. *3* 293–342, 1961.
5. *Demain, A. L.*: Cellular and environmental factors affecting the synthesis and excretion of metabolites. J. appl. Chem. Biotechnol. *22*: 345–362 1972.
6. *Demain, A. L.*: How do antibiotic-producing microorganisms avoid suicide? Annal. N. Y. Acad. Sci. *235* 601–612, 1974.
7. *Frøyshov, Ø. & Laland, S. G.*: On the biosynthesis of bacitracin by a soluble enzyme complex from *Bacillus licheniformis*. Eur. J. Biochem. *46* 235–242, 1974.
8. *Haavik, H. I. & Thomassen, S.*: A bacitracin-negative mutant of *Bacillus licheniformis* which is able to sporulate. J. gen. Microbiol. *76* 451–454 1973.
9. *Haavik, H. I.*: Studies on the formation of bacitracin by *Bacillus licheniformis*: effect of glucose. J. gen. Microbiol. *81* 383–390 1974.
10. *Haavik, H. I.*: Studies on the formation of bacitracin by *Bacillus licheniformis*: effect of inorganic phosphate. J. gen. Microbiol. *84* 226–230, 1974.
11. *Haavik, H. I.*: Studies on the formation of bacitracin by *Bacillus licheniformis*: role of catabolite repression and organic acids. J. gen. Microbiol. *84* 321–326 1974.
12. *Haavik, H. I. & Frøyshov, Ø.*: Function of peptide antibiotics in producer organisms. Nature *254* 79–82, 1975.
13. *Haavik, H. I.*: The effect of bacitracin and Mn(II) ions upon the producer strain *Bacillus licheniformis*. Acta path. microbiol. scand. Sect. B, 83 513–518, 1975.
14. *Haavik, H. I.*: On the function of the polypeptide antibiotic bacitracin in the producer strain *Bacillus licheniformis*. Acta path. microbiol. scand. Sect. B *83* 519–524 1975.
15. *Sadoff, H. L.*: Sporulation antibiotics of *Bacilli* species. Spores *5*: 157–166, 1972.
16. *Snoke, J. E. & Cornell, N.*: Protoplast lysis and inhibition of growth of *Bacillus licheniformis* by bacitracin. J. Bacteriol. *89* 415–420 1965.
17. *Weinberg, E. D.*: Biosynthesis of secondary metabolites: roles of trace metals. Adv. microbiol. physiol. *4*: 1–44 1970.
18. *Weinberg, E. D.*: Secondary metabolism: control by temperature and inorganic phosphate. Develop. Ind. Microbiol. *15* 70–81 1974.

19 *Woodruff H B.:* The physiology of antibiotic production the role of the producing organism. Soc. Gen. Microbiol. Symp. *16* 22–46 1966.

20. *Wyatt, H V.*. Cations, enzymes and control of cell metabolism. J Theoret. Biol. *6:* 441–470, 1964

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PLEURAL EFFUSION DISEASE IN RABBITS

Clinical and Post Mortem Observations

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In Denmark and probably in other countries as well, the infectious agent causing intercurrent death of rabbits by passages of Nichols pathogenic *Treponema pallidum* has been studied in rabbits in the absence of *T. pallidum*. This agent can be propagated in rabbits at intervals of 2 to 30 days and, depending on the interval between passages and the number of passages, the mortality may vary from zero to almost 70 per cent. Based on the *post mortem* findings in fatal cases, the name pleural effusion disease is suggested for this rabbit infection. Iridocyclitis, haematological and biochemical changes are signs of the disease not described previously. The source of the infectious agent is discussed.

Key words: Pleural effusion disease, rabbits, treponemal immobilization test.

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Pleural effusion disease is an infection of laboratory rabbits which apparently has been found to occur "naturally" only among rabbits inoculated with Nichols pathogenic *Treponema pallidum*. The disease was first reported in 1966 by *Jorgensen* (7) as 'spontaneous deaths among rabbits inoculated with *T. pallidum* less than 2 weeks before'.

The treponemal immobilization test (TPI) was introduced at Statens Seruminstitut in Copenhagen after the Nichols pathogenic strain of *T. pallidum* had been received in 1951 from Johns Hopkins Hospital, Baltimore, USA. From 1951 this strain was propagated in rabbits by intratesticular inoculation at intervals of 6–10 days. Until 1961 the annual incidence of death among inoculated rabbits was below 2 per cent, but from 1961 to 1971 this incidence rose gradually from 2 to 38 per cent.

The Nichols strain was sent from Copenhagen to the TPI laboratories in Stockholm and Oslo in 1953 and 1957 respectively. In these laboratories, the same type of intercurrent death among inoculated rabbits was recognized in 1962 (2, 3, 4). In 1971 a similar mortality phenomenon occurred at Institut Alfred Fournier in Paris (11). This institute had maintained a Nichols

cured directly from Baltimore in 1950 Inter current death had been negligible In 1971 the mortality among inoculated rabbits suddenly increased from 3 to 35 per cent within 4 weeks. Unexpected mortality also seems to have occurred in TPI laboratories in Finland and Spain after the introduction of the Nichols strain from Copenhagen (8)

The clinical picture and *post mortem* findings of the intercurrent disease were described by *Jørgensen* (7) and *Gudjónsson & Skog* (3 4) So far the aetiology remains obscure but, according to *Gudjónsson et al.* (5 6) it is a virus-like agent which apparently is passed from rabbit to rabbit together with the treponemes. The removal of this agent from the Copenhagen and Stockholm substrains of the Nichols strain was reported by *Skovgaard Jensen* (9) and *Gudjónsson et al.* (6) respectively

Studies of the infection caused by the unknown agent in rabbits in the absence of *T pallidum* have been carried out. This paper presents the results of the passages of this infectious agent in rabbits over a period of 3 years. In addition, a group of rabbits were infected in order to study various aspects of the disease in more detail.

MATERIAL AND METHODS

Rabbits All animals were random-bred conventional albino rabbits (Ssc:CPH) from Statens Seruminstitut. The rabbits, weighing 1800–400 g, were fed a pelleted diet supplemented with hay and water and kept in solid cages. For a final experiment, male rabbits had been raised on wire mesh bottoms and, during the experiment, they were kept in plastic cages and fed a pelleted diet and water *ad libitum*. At the time of inoculation, these animals were 91–92 days old and weighed 2200–2700 g.

Animal passages. Propagation of the agent was started on October 22, 1970 by subcutaneous inoculation of a freeze dried testicular suspension of treponemes in saline solution into a female rabbit. The lyophilization is considered to cause death of pathogenic treponemes (10) The treponemes originated from two rabbits sacrificed 10 days after intratesticular inoculation of the Nichols strain. After 32 continuous passages in female rabbits at intervals of 2–14 days, systematic propagation in male rabbits was started on March 13 1971 These passages, each comprising one animal, were made by subcutaneous inoculation of 1 ml ear blood or, in case of death before day 10, by inoculation of 1 ml of pleural fluid or heart blood. In this way a total of 170 passages over a period of 3 years were made at intervals of 3–10 days. From the 85th passage, a new parallel line of passages was carried out in a similar manner but at an interval of 20 days in surviving animals propagation was continued for 1 year totalling 24 passages. A third line of passages was started from the 121st passage at intervals of 30 days, lasting for 1 year and totalling 12 passages. From the 142nd passage, a fourth and a fifth parallel line of passages were attempted at intervals of 40 and 60 days, respectively

Observations concerning animal passages. Inoculated rabbits were observed for 2–4 weeks with a view to onset of fever and other clinical signs of disease. Dead animals were examined bacteriologically by seeding blood agar plates with material from heart blood, lung liver spleen and mesenteric lymph nodes. Gross lesions were noted, the lungs were weighed and liquid from the pleural space, occasionally also from the peritoneal cavity was collected and measured.

All animals used for passages at intervals of 30, 40 and 60 days were challenged at the end of the observation period by subcutaneous inoculation of 1 ml of pleural fluid of known infectivity in order to ascertain whether the animals were immune.

Infection experiment A final experiment comprising 12 rabbits was carried out. During a preperiod of 8 days, these animals were observed for signs of disease blood was examined twice for total and differential leucocyte counts, erythrocyte count, haemoglobin concentration, packed cell volume, Na and K contents, lactic dehydrogenase (LDH) urea, glucose total plasma protein and protein fractions. After inoculation, the animals were followed closely and examined for the same parameters for a period of up to 6 weeks. Blood for these examinations was always drawn at the same hour each day Dead animals were autopsied within 3 hours after death and pleural fluid was examined for cells and total protein content. Survivors were examined after sacrifice on day 48.

Inoculum. Each animal was infected subcutaneously with 1 ml of a pool of pleural fluid diluted 1 10. This pool containing about 10^4 rabbit infective doses per ml, originated from 11 animals that died on the 3rd day after inoculation during the 80th to 163rd passage of the agent.

Statistical analysis. The distributions of the clinical responses are all skew and therefore Wilcoxon's Two-Sample Test* was used for the significance tests.

* See e.g. DOCUMENTA GEIGY Scientific Tables, 6th Ed., 1962, p. 191

As regards the infection experiment it was found for each of the characteristics that the logarithm of the observations could be regarded as normally distributed. As to the group of survivors, a two-way analysis of variance was carried out and the residual variance was used for the calculation of the standard errors of the means plotted in Fig 2.

RESULTS

Clinical Response in Animal Passages

Tables 1 and 2 summarize the results of infection of rabbits by continuous passages of the unknown agent. In Table 1 the animals are grouped according to the interval between passages and the number of passages. Table 2 comprises only animals surviving the infection, but this group is representative also of the rabbits that died. As seen from the tables, the infection was nearly always severe when the agent was passed at 3–10-day intervals, but mild or subclinical when passed at intervals of 30 days. (Upon challenge, the "30-day interval" animals showed no clinical signs of disease, except for ephemeral fever on day 6 in three of the 12 rabbits) The difference between the three groups is significant for all the characteristics of the clinical responses except for the day on which maximum weight loss was observed. Table 1 also shows that the severity of infection clearly increased during the 3-year period of passages. One attempt to pass the agent at intervals of 40 and 60 days failed Upon challenge, the rabbit in the 2nd passage at the 40-day-interval died, presenting typical symptoms the same applied to the rabbit in the 1st passage at the 60-day-interval.

Fever The first sign of disease was usually fever i.e a rectal temperature of 40 C or more and, in the great majority of rabbits, this initial fever also represented the maximum temperature to be observed The onset

TABLE 1 *Mortality and Time of Death During Passages in Rabbits*

Passage nos. (duration)	Interval between passages (days)	Mortality (per cent)	Number of deaths after inoculation (per cent)		
			1st week	2nd week	3rd week
1-61 (1st year)	3–10	24/61 (39)	15 (62)	9 (38)	0
62-111 (2nd year)	3–10	24/50 (48)	18 (75)	5 (21)	1 (4)
112-170 (3rd year)	3–10	40/59 (68)	35 (87)	4 (10)	1 (3)
1 24 (one year)	3–20	9/24 (36)‖	4 (44)	4 (44)	1 (12)
1 12 (one year)	30	0/12 (0)		none	

Four cases of intercurrent death counted as survivors.
‖ Mortality for 3–10 days passages in comparable period 51 per cent.

TABLE 2 *Clinical Response in Surviving Rabbits*

Number of animals	Interval between passages (days)	Fever			Eye changes			Weight loss		
		number	onset (day)	duration (days)	number	onset (day)	duration (days)	number	maximum loss (grammes)	on day
[illegible]	3–10	68	1–3 (2)	1 22 (9)	78	1 5 (2)	6–29 (15)	75	20–370 (180)	20 (4)
[illegible]	3–20	14	2–4 (2)	1 16 (8)	15	2 10 (3)	3–19 (12)	14	10–300 (150)	3–12 (4)
[illegible]	30	4	2–4 (2)	1 9 (2)	5	4–10 (6)	1–18 (9)	10	10–180 (60)	2 14

Figures in parentheses are median values.
day indicates day after inoculation.

of fever was followed by a febrile period with irregular fever of varying duration. In more than one third of the survivors, this fever was of the diphasic or triphasic type. A lytic fall below 38 C, lasting for 1–4 days, was seen in about 40 per cent of the fatal cases.

Eye changes * Nearly all the rabbits showed characteristic eye changes. These changes started with distension of the episcleral and conjunctival vessels in the dorsal and ventral part of the eyes and were followed by a marked hyperaemia of the iris. A few animals showed oedematous eyelids or small haemorrhages in the anterior chamber. Slitlamp examination of two typical cases (day 7) revealed marked hyperaemia of the iris, diffuse aqueous flare and dust like precipitates on the back of the cornea and, in one of the cases, small adhesions between the iris and the lens. A pool of anterior eye chamber fluid, originating from 10 rabbits and drawn on the 3rd day showed a protein content of 1.25 per cent, i.e. a considerable increase compared with the normal value of about 0 1 per cent.

Weight loss Loss of weight occurred in most animals. The lowest weight was usually recorded during the 1st week but in many animals weight gain did not start until 2–4 weeks after inoculation

Other symptoms In a small proportion of animals, particularly among those that died a transient tachypnoea was noted on the 2nd or 3rd day

Death Death occurred as early as the 3rd day and as late as the 17th day The mortality was concentrated on the 3rd-4th day (67 per cent) but another peak occurred on the 9th 10th day (16 per cent)

Post mortem Findings in Animal Passages

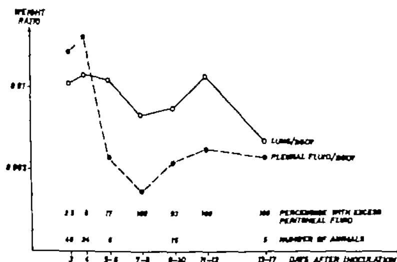
Based on autopsy findings and the results of bacteriological and parasitological examinations it is considered that 97 of the 101 rabbits had died from the unknown infection. Four animals, dying on day 4 5 or 8, were considered to have died from other causes (one case of liver rupture, two of intestinal invagination and one case of *Clostridium welchii* septicaemia) Ninety-one of the 97 animals showed a fairly uniform picture. The lungs were regularly oedematous and hyperaemic and the bronchi were usually filled with a frothy content. The right part of the heart was often dilated and the pleural cavities contained 2–50 ml of a serous, slightly opaque fluid with or without fibrin and with very few cells. In addition, almost all the animals dying after the 1st week had increased amounts (10–200 ml) of a fluid of similar appearance in the peritoneal cavity In many of these animals, the liver was moderately enlarged and the spleen slightly enlarged. In six animals, no increase in the amount of pleural fluid was found, but lung oedema was manifest in four of these (dying on day 5 6 or 10) and in one (dying on day 13) the amount of peritoneal fluid was increased. Only one animal (which died on day 4, presenting typical symptoms) did not show any of these changes.

Fig 1 shows the relation between lung weight and pleural effusion at various times of death after inoculation. The figure also shows the percentage of animals with peritoneal effusion. Unfortunately the amount of fluid in the peritoneal cavity was not measured systematically but the few available observations suggest that the amount of peritoneal fluid increased with time after inoculation.

Infection Experiment

Clinical response On the 2nd day after inoculation, all 12 rabbits showed a rise in temperature (range 39 7–41.0 C, average 40.3 C) and eye changes. Two rabbits also showed tachypnoea. Seven animals died 54–67 hours after inoculation. In the remainder the febrile period lasted for 9–26 days (average 15 days) and the eye changes persisted for 8–19 days (average 12 days) In these animals, the weight loss ranged from 220 to 430 g the minimum weights were recorded during the interval from the 4th

* The eye changes will be described in a separate communication.



I Arithmetic means of lung/body and pleural fluid/body weight ratios, and percentage of animals h excess peritoneal fluid.

TABLE 3. *I f ction Experiment Post mortem Findings in Fatal Cases*

Rabbit no.	Body wt. kg	Lung wt. g	Pleural fluid ml	Pleural fluid protein g/100 ml	Proteinuria
1	2.2	23	23	5 1 (7.2)	+
2	2.7	32	31	6.6 (9.9)	trace
7	2.3	15	29	4.9 (8.6)	trace
8	2.8	27	24	5 4 (8.7)	0
9	2.3	12	2	6.6 (9.3)	trace
10	2.3	26	26	5.6 (8.6)	+
11	2.6	23	25	6.8 (7.9)	trace

Figures in parentheses are corresponding plasma alues.

the 10th day but weight gain did not start ntil the 13th day continuing until the 23rd y Water intake declined from the 2nd y and was not normal until the 14th to th day

Haematological and biochemical findings. he leucocyte response was characterized by slight, transient leucopenia due to a reduc on in the number of lymphocytes at the me of the first symptoms. In the survivors, ese early changes were followed by a leucocytosis due to an increase in the number of eterophils. At the time of the first symptoms ere was also a definite fall in erythrocyte ount, packed cell volume and haemoglobin concentration which reached a minimum on day 7 in the survivors.* On day 42, the blood picture was again almost normal (Fig 2 a and b)

A decrease in total plasma proteins with a corresponding fall in albumin occurred dur ing the 2nd-3rd week. At the end of the observation period, the concentration of gamma globulin had increased significantly Serum potassium showed a slight rise continuing until day 21 but was almost normal on day

* The slight fall in these alues prior to inocul tion is onsidered to be due to the amount of blood drawn.

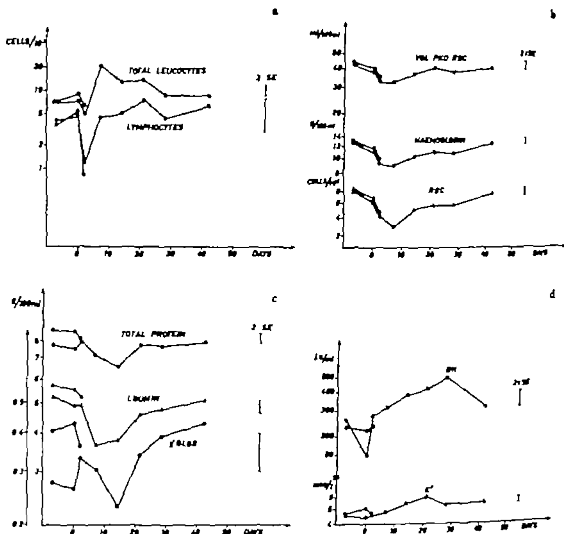


Fig 2 Infection experiment. Day 0 indicates day of inoculation. Ordinates All values are drawn on a logarithmic scale. The short curves represent seven rabbits that died on day 5 and the long curves the five survivors. VOL. PKD RBC = Volume packed red blood cells. RBC = Red blood cells. LDH = Lactic dehydrogenase

28. Serum lactic dehydrogenase (LDH) showed a slight rise which continued until day 28 (Fig. 2 c and d)

Serum sodium blood urea and glucose did not show any particular changes, except for a transient rise in blood urea in two animals on day 7 (74 and 103 mg per cent)

Post mortem findings Table 3 gives a survey of some of the findings in the fatal cases. As will be seen, the protein concentration in the pleural fluid approached the plasma value The total cell count of the pleural fluid was below 200 per cmm In six out of seven animals examined.

The survivors showed no obvious gross changes of sacrifice. In one animal, a slight degree of hepatic coccidiosis was noted and all five animals had proteinuria (trace to + + + Albustix, Ames Co.) The histopathological changes will be described in a separate communication.

DISCUSSION

The present investigation shows that the infectious agent contaminating the Copenhagen substrain of Nichols pathogenic *T. pallidum*, and presumably other Nichols substrains, can easily be propagated in rabbits without treponemes. The agent causes disease and mortality rates similar to those observed during propagation of the contaminated Nichols substrains (2, 3, 4, 7). The occurrence of iridocyclitis and of distinct haematological and biochemical changes has not been described previously. These signs are compatible with the suggestion that it may be a virus-like agent (5, 6). The reason why *Gudjónsson* & *Skog* (4) failed to detect a leucocyte response in 20 rabbits may be that their rabbit material was heterogenous.

The *post mortem* findings after early death were characterized by pleural effusion and lung oedema and after late death, *i.e.* after the 1st week of infection, also by peritoneal effusion and liver enlargement. In cases of naturally occurring rabbit infections this picture has apparently not been observed and the name pleural effusion disease seems to be the appropriate term to apply to this "new" rabbit disease.

Short intervals (3–10 days) between passages and increase in the number of passages augmented the virulence of the agent, whereas longer intervals (30 days) resulted in almost subclinical infection. This latter observation indicates that a Nichols strain carrying the agent as "passenger" may well be propagated for at least 1 year without obvious disease and mortality provided that the intervals between passages are sufficiently long.

As yet, the source of the causative agent remains obscure, but the history and epidemiology of the disease together with the present results and the circumstantial evidence suggest that the agent was imported to Copenhagen with the Nichols strain procured from Baltimore in 1931. About 10 years later a mortality problem presented itself in the TPI laboratories in Copenhagen, Stockholm and Oslo, *i.e.* after a comparable number of rabbit passages of this Nichols strain in the Scandinavian countries. The lag period between import of the strain and the time when the mortality problem turned up may be explained by the subclinical presence of the agent in the rabbit passages during this period.

Admittedly this does not prove that the Nichols strain received in Copenhagen in 1951 was contaminated. On the other hand, rabbits infected in Baltimore in 1970 with the local Nichols strain apparently developed immunity to subsequent challenge with the contaminated Nichols strain from Stockholm (6).

It may be argued that the infectious agent might as well have originated from the rabbit colony supplying animals for the propagation of the treponemes. The following observation at Statens Seruminstitut, Copenhagen does not support this hypothesis. In the period from 1954 to 1967 the Brown-Pearce carcinoma was maintained in rabbits by intratesticular inoculations at a rate of about five rabbits per week. In the same period, the mortality problem occurred among the treponema inoculated rabbits. Nevertheless, a mortality problem resembling that involved in pleural effusion disease was never encountered during these 14 years of passages (1). The number of rabbits used for the passage of the Brown-Pearce carcinoma was of the same order as that used for the propagation of the Nichols strain, and the rabbits were from one closed rabbit colony which has been [illegible]. It is therefore unlikely that the agent present in the passages of the Nichols strain was introduced from this source.

The [illegible] Paris where the mortality problem [illegible] 20 years after the Nichols strain had been procured [illegible] from Baltimore [illegible] might also serve [illegible] strain [illegible] contaminated [illegible] infection [illegible]. The failure to [illegible] Nichols strain [illegible] laboratories [illegible] understandable [illegible] length of the [illegible]-free periods [illegible] passages [illegible]

MATERIALS AND METHODS

Design of the Study

The study was carried out as a prospective study during the period January 1st to December 31st, 1973 All strains of *P aeruginosa* isolated from the routine bacteriological specimens received in the Department of Clinical Microbiology at Blegdamshospitalet were examined on routine media (8) for the presence of mucoid colonies. Identification of *P aeruginosa* and of mucoid colonies was done according to published criteria (4 7 8, 11) and all strains were subcultured on the standard differential medium for gram-negative rods, supplied by Statens Seruminstitut (8) at 35 C for 48 h as the production of mucoid substance is abundant on this medium (unpublished observation) If mucoid colonies were present in the initial cultures and/or in the subcultures, then the strain was classified as mucoid, although non-mucoid variants or dissociants sometimes were present simultaneously in the same specimen (1) If mucoid strains were isolated in one or more specimens from a pt., then the pt. was classified as harbouring mucoid strains, although *a*) non-mucoid variants sometimes were present simultaneously in the same specimen, and *b*) the pt. might harbour solely non-mucoid strains in subsequent specimens (9)

Patients

The Department of Clinical Microbiology receives specimens from hospitalized pts. and out pts. treated in the Department of Infectious Diseases, including tracheotomized pts. suffering from severe respiratory failure the Department of Internal Medicine, the Department of Oto-rhino-laryngology Blegdamshospitalet, and the Paediatric Clinic TG with the Paediatric Clinic of Dronning Louises Børnehospital, Rigshospitalet, the centre of CF treatment in Denmark.

Statistical Methods

The χ^2-test and Student's t-test. Level of significance 5 per cent (double-tailed tests)

RESULTS

During 1973 18 444 bacteriological specimens were received. *P aeruginosa* was isolated from 1054 (5 7 per cent) of the specimens originating from 222 pts. (Table 1) The pts. comprised 26 males and 27 females with cystic fibrosis (mean age 10 years, range 8 months—26 years) and 103 males and 66 females with other diseases (mean age 54 years, range 14 days—96 years)

Table 1 shows the distribution of the *P aeruginosa* strains on pts. with CF and pts. with other diseases. All the strains from CF pts. were isolated from the respiratory tract (99.5 per cent from sputum or laryngeal secretion) The relative prevalence of mucoid strains was much higher in specimens from CF pts. than in specimens from pts. suffering from other diseases ($p < 0.0005$) both with respect to percentage of isolates and percentage of pts. harbouring the strains. Mucoid and non-mucoid strains were alternatively isolated from subsequent specimens obtained from each of 28 of the CF pts. and 5 of the non-CF pts. The simultaneous presence of mucoid and non-mucoid variants in some of the specimens was also noted and will be reported elsewhere (1) The relative prevalence of mucoid strains was similar in the two sexes. Within each of the two groups of

TABLE 1 *Prevalence of Mucoid Strains of* Pseudomonas aeruginosa *in Clinical Specimens from Patients with Cystic Fibrosis and Patients with other Diseases*

	Total No of *P aeruginosa* strains	No. of mucoid strains	Total No. of patients harbouring *P aeruginosa*	No. of patients harbouring mucoid strains
Patients with cystic fibrosis	551	439 (80 %)	53	45 (85 %)
Patients with other diseases	503	15 (3 %)	169	10 (6 %)
Total	1054	454 (43 %)	222	55 (25 %)

No. number

TABLE 2. *Site of Isolation of 503 Strains of* Pseudomonas aeruginosa *from Patients with other Diseases than Cystic Fibrosis*

Origin of strains	Total No. of *P. aeruginosa* strains	No. of mucoid strains	Total No. of patients harbouring *P. aeruginosa*	No. of patients harbouring mucoid strains
Respiratory tract	283	4 (1.4 %)	84	3 (3.6 %)
Urogenital tract	79	7 (8.9 %)	47	4 (8.5 %)
Middle ear	81	3 (3.7 %)	45	3 (6.7 %)
Wounds	28	0	14	0
Blood cultures	11	1 (9.1 %)	5	1
CSF, eyes, faeces*	21	0	4	0

As a patient can harbour *P. aeruginosa* in more than one region, the total No. of patients amounts to more than the 169 given in Table 1. No.: number.
* Faeces was very seldom investigated for *P. aeruginosa*. CSF: cerebro spinal fluid.

pts. there was no significant difference as to age between pts. harbouring mucoid strains and pts. harbouring non-mucoid strains of *P. aeruginosa*.

Table 2 shows the strains isolated from non-CF pts. distributed according to anatomical origin of specimens. It is seen that mucoid strains were infrequently isolated in specimens from all anatomical regions, including sputum and secretion obtained by endo-laryngeal suction, which represented 86 per cent of the specimens with *P. aeruginosa* obtained from the respiratory tract.

DISCUSSION

In accordance with findings by other authors the results show a pronounced difference between the relative prevalence of mucoid strains of *P. aeruginosa* in specimens from CF pts. and in specimens from pts. suffering from other diseases (2, 3, 5, 12). Alternation between mucoid and non-mucoid strains of *P. aeruginosa* in subsequent specimens as well as the simultaneous presence in some specimens of mucoid and non-mucoid variants were seen in many of the pts.—in CF pts. as well as in non-CF pts.—as reported elsewhere (1, 9). Moreover, the results show that mucoid strains of *P. aeruginosa* show no preference for the respiratory tract except in CF pts.

The nature of the selective factors favouring mucoid strains in the respiratory tract of CF pts. is not clear, but several possibilities including CF specific factors and the pronounced humoral immune response have been proposed as discussed in detail elsewhere (1–7, 9, 10, 12).

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REFERENCES

1. *Bergan, T. & Høiby, N.*: Epidemiological markers for *Pseudomonas aeruginosa*. 6. Relationship between concomitant non-mucoid and mucoid strains from the respiratory tract in cystic fibrosis. Acta path. microbiol. scand. Sect. B, *83*: 553–560, 1975.
2. *Burns, M. W.*: Significance of *Pseudomonas aeruginosa* in sputum. Brit. Med. J. *3*: 382–385, 1973.
3. *Diaz, F., Mosovich, L. L. & Neter, E.*: Serogroups of *Pseudomonas aeruginosa* and the immune response of patients with cystic fibrosis. J. Infect. Dis. *121*: 269–274, 1970.
4. *Doggett, R. G., Harrison, G. M., Stillwell, R. N. & Wallis, E. S.*: An atypical *Pseudomonas aeruginosa* associated with cystic fibrosis of the pancreas. J. Pediatrics *68*: 215–221, 1966.
5. *Doggett, R. G.*: Incidence of mucoid *Pseudomonas aeruginosa* from clinical sources. Appl. Microbiol. *18*: 936–937, 1969.

6. *Doggett R G & Harrison G M Pseudomonas aeruginosa* Immune status in patients with cystic fibrosis. Infect. and Immun. *6* 628–635 1972.
7 *Høiby N & Axelsen N H.*. Identification and quantitation of precipitins against *Pseudomonas aeruginosa* in patients with cystic fibrosis by means of crossed immunoelectrophoresis with intermediate gel. Acta path. microbiol scand. Sect. B, *81* 298–308 1973
8. *Høiby N.*. Epidemiological investigations of the respiratory tract bacteriology in patients with cystic fibrosis. Acta path. microbiol. scand. Sect. B, *82* 541–550 1974
9 *Høiby N Pseudomonas aeruginosa* infection in cystic fibrosis. Relationship between mucoid strains of *Pseudomonas aeruginosa* and the humoral immune response. Acta path. microbiol. scand. Sect. B, *82* 551–558, 1974
10 *Høiby N., Andersen V & Bendixen, G.*. *Pseudomonas aeruginosa* infection in cystic fibrosis. Humoral and cellular immune responses against *Pseudomonas aeruginosa*. Acta path. microbiol. scand. Sect. C, *83* 459-468, 1975
11 *Jessen O.*. *Pseudomonas aeruginosa* and other green fluorescent pseudomonads. Thesis, Munksgaard, Copenhagen 1965, p. 61
12. *Martin D R.* Mucoid variation in *Pseudomonas aeruginosa* induced by the action of phage. J Med. Microbiol. *6* 111–118, 1973.

Acta path. microbiol. scand. Sect. B, 83 553–560, 1975

EPIDEMIOLOGICAL MARKERS FOR *PSEUDOMONAS AERUGINOSA*

6. Relationship between Concomitant Non-mucoid and Mucoid Strains from the Respiratory Tract in Cystic Fibrosis

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Bergan, T & Høiby N Epidemiological markers for *Pseudomonas aeruginosa*. 6 Relationship between concomitant non-mucoid and mucoid strains from the respiratory tract in cystic fibrosis. Acta path. microbiol. scand. Sect. B *83* 553–560 1975.

The simultaneously occurring mucoid (M) and non-mucoid (NM) variants of *Pseudomonas aeruginosa* frequently observed in cultures from the respiratory tract of chronically infected cystic fibrosis patients have been studied. M cultures *in vitro* were unstable and easily dissociated NM colonies. In a large proportion of the cases, M and NM variants occurring simultaneously in cultures from one and the same clinical specimen were of the same pyocine type, phage type, and serogroup. In some remaining cases there were small differences between the M and NM variants such that identity between the variants from one and the same specimen was possible, although not definite. The NM dissociants from M strains were of the same type as the M variant. The possible role of cross-infection and the interaction of bacteria and host response factors continuously tending to select the unstable M variant *in vivo* are discussed.

Key words. *Pseudomonas aeruginosa* epidemiological markers serotyping pyocine typing bacteriophage typing cystic fibrosis.

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Mucoid cultures of *Pseudomonas aeruginosa* are frequently isolated from the respiratory tract of patients with cystic fibrosis (CF) (7 10, 11 14 16) Non-mucoid isolates from the same culture are regularly seen (9 12) Prospective studies have indicated that non-mucoid (NM) variants often precede mucoid (M) variants (10, 12, 15) This could follow nosocomial superinfection, since coexistence of strains of different epidemiological types has been a common finding in chronic infections both in CF and non-CF patients (4 9) Lysogenization may also be responsible since NM variants after uptake of prophage may be transformed into mucoid strains producing abundant polysaccharide (1 8 21) It has also been suggested that the selection of M variants in CF may be enhanced by immune host mechanisms (15 17 18)

TABLE 1 *Source of Mucoid (M) and Non-mucoid (NM)* Pseudomonas aeruginosa *Isolated f*
Typing Results of Pyocine Ty

Patient	Age	Sex	Strain	M/NM morphology Cph.	Tromsø	Isol.-dat
T B.	9	M	452	NM	NM	28/3
			452	M	M	28/3
			1155	NM	NM	21/11
			1155	M	M	21/11
			1155	M	NM	21/11
M. W S	6	M	458	NM	NM	23/3
			458	M	M	23/3
			458	M	NM	23/3
			815	NM	NM	30/7
			815	M	M	30/7
			815	M	NM	30/7
H. T N	7	F	490	NM	NM	4/4
			490	NM	M	4/4
			490	M	M	4/4
			1082	NM	NM	24/10
			1082	M	M	24/10
			1082	M	NM	24/10
B. F S K.	15	M	495	NM	NM	11/4
			495	M	M	11/4
			495	M	NM	11/4
C. H. C.	15	M	685	NM	NM	6/6
			685	M	M	6/6
			685	M	NM	6/6
			1168	NM	NM	21/11
			1168	M	M	21/11
S. E. J	22	M	904	NM	NM	22/8
			904	M	M	22/8
			904	M	NM	22/8
			1218	NM	NM	12/12
			1218	M	M	12/12
			1218	M	NM	12/12
			1258	M	M	30/12
			1258	NM	M	30/12
			1258	NM	NM	30/12
P Lu.	9	M	955	NM	NM	28/8
			955	M	M	28/8
			955	M	NM	28/8
			1115	M	M	6/11
			1115	NM	M	6/11
			1115	NM	NM	6/11
J P	18	F	1022	NM	NM	3/10
			1022	M	M	3/10
			1022	M	NM	3/10
B. J M.	7	F	1081	M	M	24/10
			1081	NM	NM	24/10
P La.	7	M	1099	NM	NM	29/10
			1099	M	M	29/10

II

...nts with Cystic Fibrosis Colony M rphology (M or NM) in Copenhagen (Cph.) or Tromsø and ...p Typing, and Serogrouping*

...me type	Phage type	Serogroup	Comments	Ambulant Hospitalized
1,3,7 8	7 18, 19	9	NM & M identical	A
1,3,7 8	7 18, 19	9		
1,3,7	19	9	NM & M identical	A
1,3,7	12, 19	9	a	
1,3,7	2,5 12, 19 21	9		
7	2,5 12, 16, 19 21	9	NM & M identical	H
7	2,5 12, 16 19 21	9	a	
7	2,5 12 16 19 21	9		
7	2,5, 6, 12, 16, 18, 19 21	9	NM & M identical	H
7	2,5 6, 12, 16, 18, 19 21	9	a	
7	2,5,6 12, 16, 18, 19 21	9		
1,3,4,5,7 8	2, 12, 19	9	NM & M non-identical	A
5,7	2, 12, 19	9	b, c	
7	2, 12, 19	9		
1,3,4,5,7 8	19	2	NM & M identical	A
1,3,4,5,7 8	19	2	a	
1,3,4,5 7 8	19	2		
7	2, 13, 19	3	NM & M identical	A
7	2, 8, 13 19	3		
7	2, 19	3		
2,5,6,7	5, 12, 19 21	3	NM & M identical	A
7	5 12, 18, 19 21	3	a	
2,5,6,7	5 12, 19 21	3		
	12 19 21	3	NM & M identical	H
	12, 19 21	3		
5				
2,3,5,6,7	2,5 12, 19 21	9	NM & M incompl identical	A
2,3,5,6,7	2,5 12, 19 21	9	a	
5,6	2,5 6, 12, 19, 21	9		
2,3,5,6,7	5 12, 19 21	9	NM & M identical	A
2,3,5,6,7	5 19 21	9	a	
7	5 12 19 21	9		
	19	9	NM & M non-identical	H
2,3,5,6,7	5 12, 19 21	9	a, d	
2,3,5,6,7	5 12, 19 21	NT		
2,3,5 7	1 19	6	NM & M identical	H
2,3,5,7	1 19	6	a	
2,3,5,7	1 19	6		
2,3,5,7	19	NT	a, c, d	H
2,3,5,7	19	NT		
2,3,5 7	19	6		
2,3 5,7	19	6	NM & M identical	A
2,3 5,7	19 21	6	a	
2,3 5 7	19 21	6		
,7	19 21	9	NM & M non-identical	A
,5,7	2,5 12, 19 21	9	d	
,3,5,7	19	5	b	A
,7	19	NT		

T/

Patient	Age	Sex	Strain	M/NM morphol. Cph.	Tromsø	Isol.-da
P La.			1138	NM	NM	14/11
			1138	M	M	14/11
			1138	M	NM	14/11
L N	7	F	1101	NM	NM	31/10
			1101	M	M	31/10
			1101	M	NM	31/10
J F	6	F	1118	M	M	7/11
			1118	NM	NM	7/11
M. L. M.	2	F	1139	NM	NM	14/11
			1139	M	M	14/11
			1139	M	NM	14/11
J A.	11	F	1203	NM	NM	5/12
			1203	M	M	5/12
			1203	M	NM	5/12

) Twenty-two pairs of mucoid and non-mucoid strains isolated from 22 different specimens of sputum or laryngeal aspirates from 14 patients with cystic fibrosis. The successive specimens from seven of the patients were obtained at intervals of from 16 days to 8 months. Six of the pairs of M and NM bacterial strains were obtained from hospitalised patients sixteen from outpatients.

a) M variant in Cph. which in Tromsø rendered NM dissociant of identical epidemiological type

b) M and NM variants may possibly be of one and the same epidemiological type.

The purpose of the present study is to elucidate the relationship between concomitantly occurring M and NM variants by way of pyocine typing, bacteriophage typing and serogrouping

MATERIALS AND METHODS

Bacterial Material

The bacteria originated from a group of approximately 100 CF patients who regularly every 1–2 months, appeared in the Paediatric Outpatient Clinic TG Rigshospitalet Tagensvej Copenhagen. This institution serves as the centre of CF treatment in Denmark. As part of the examination routine, specimens for bacteriological cultivation were taken from sputum or by endolaryngeal suction (14) of secretions.

During 1973 72 cultures rendered mucoid (M) as well as non-mucoid (NM) variants of *P aeruginosa*. These were isolated by procedures reported elsewhere (14) and identified according to *Jessen* (19) on the basis of pyocine and fluorescein production on King's special media and reduction of nitrate.

The M and NM variants were stored separately at room temperature in meat extract agar slants with sealed corks. After ½–1½ years, one to three subcultures were made for the segregation of dissociants int pure cell lines before shipment on slants to Tromsø for typing. At that time, 39 of the 72 originally mucoid strains had changed to NM. The remaining 33 pairs of M and NM variants were shipped during early fall, before outdoor freezing temperatures started. After 1½ months, subcultures were made on blood agar (Tryptose Blood Agar Base (Oxoid) with 5 per cent human blood) at 37 C in a saturated, humid atmosphere overnight. The number of cultures which on arrival in Tromsø still rendered both M and NM dissociants was 22. These were derived from the lower respiratory tract of 14 chronically infected CF patients, the key characteristics of whom are given in Table 1 Up to three specimens were taken from each subject during intervals of from 16 days up to 8 months.

Colony morphology was studied carefully (above cultural conditions) and cloned 2-4 times to ensure stability of the M or NM character before typing

…nt.)

…cin type	Phage type	Serogroup	Comments	*Ambulant* *Hospitalized*
…3,7	2, 19	9	NM & M non-identical	A
…7	2, 19	3	a	
…7	2, 19	3		
…2,3,4,5,6,7 8	12, 17 19 23	5	NM & M identical	A
…2,3,4,5,6,7 8	12, 19 21	NT	a	
…2,3,4,5,6,7 8	12, 19 21	5		
…7	2, 5, 12, 16, 19 21	NT	NM & M identical	A
…7	2 5 12, 16, 19 21	9	d	
…7	2, 5, 12, 16, 19 21	9	NM & M non identical	A
…7	19	NT	a	
…3,7	2, 5 12, 19 21	9		
…7	2, 19	3	NM & M non-identical	A
…3,7	8, 9	NT		
…7	19 21	NT		

c) NM variant from Cph. which in Tromsø rendered M dissociants of an epidemiological type other than NM, though of the same type as M in Cph.

d) M and NM variants in Cph. turned out as NM and M respectively when they were first cultivated in Tromsø. This was presumably caused by an error in the marking of cultures accordingly M and NM markings were adjusted in the table.

e) M variant from Cph. which in Tromsø rendered NM dissociants. These belonged to a different epidemiological type.

Typing Procedures

1. *Bacteriophage typing* procedures were as previously described (2)

The typing set consisted of the phages 73 F7 M5, Ms13 113 F116, Px3 O1c, C4 C13 C21 F2/49, F10, VII, Z2, Z3, Z19 and Z20 as the primary typing set and the phages 21B, 68 Col 11 K9 and C15 as an auxiliary typing set. For convenience, these strains are in the text and in the tables referred to as numbers 1–19 and Aux 1 to Aux-5 in the order indicated above.

2. *Pyocine typing* was carried out by the indicator set and method of *Gillies & Govan* (13) with minor modifications (3)

3. *Serogrouping* was done by slide agglutination with live antigens from overnight blood agar cultures. Antigens of mucoid and non-typable strains were prepared by boiling for 2 ½ hours followed by washing twice in 0.85 per cent sterile saline. The antigens and grouping procedures have been detailed previously (3)

The pairs of M and NM variants were typed blind without knowledge of patient identity on the part of the typing laboratory

Terminology

Although there is no fundamental difference, in order to achieve better distinction in presentation, the M and NM strains isolated from the first subculture will in this paper be referred to as *variants* whereas cell lines emerging from these upon subcultivation are to be called *dissociants*. When from an M variant, an NM dissociant of the same epidemiological type appears, this will be referred to as M NM-variation.

RESULTS

Colony Variation

Here the emphasis is on the type relationship between M and NM variants occurring in the same specimens. Accordingly an initial study of the colony morphology during serial subcultures was essential. In 18 of the 22 mucoid cultures, NM dissociants emerged* (Table 1) Three of the strains which … penhagen had been found to be non

rendered also mucoid cell lines, upon arrival at the typing laboratory It is to be noted that careful cloning had been carried out immediately before shipment in order to safeguard that the cultures were pure at that time. In some istances, the first subculture in Tromsø of M variants rendered entirely mucoid colonies, only to produce NM dissociants upon further subcultivation. Similar experience was made in Copenhagen.

These results point to an instability of the mucoid character It is notable that change from NM to M was rare. One may wonder whether the cell line actually had been contaminated throughout with a few M variants, the detection of which was suppressed by overgrowth by the NM variant.

Typing of M and NM Strains from One and the Same Specimen

The results of pyocine typing phage typing, and serogrouping of pairs of both M and NM variants from one and the same specimen appear in Table 1 In 13 of the 22 cases, the typing patterns were compatible with strain identity both of the M and the NM strains and their dissociants. The specimen no. 904 showed identity in phage type and serogroup but the pyocine type differed. There was complete identity between the M variant and NM dissociants derived from it.

Similarly both variants and one M dissociant from NM of specimen 490 were identical in phage type and serogroup but differed in pyocine type. The strains from specimen 1258 exhibited a similar pattern. As regards specimen 1138 the M variant and the NM dissociant from it showed type identity whereas the variant which in Copenhagen had been identified as NM belonged to another serogroup. Two series of strains (nos. 1099 and 1115) were of slightly varying types, but considering the permissible variation in typing codes, identity of the isolates is entirely possible—although not definite. Regarding the strains nos. 1139 and 1203 the typing patterns were not compatible with identity of the M and NM variants. In 13 instances, M variants rendered NM dissociants of the same type.

The eleven pairs of strains, which upon arrival in Tromsø, not included in Table 1 were of identical colony types, comprised two pairs where both strains were mucoid together with nine pairs of NM variants. The former were serologically untypable, whereas the remainder belonged to serogroup 9 and, according to pyocine type and phage type belonged to the most frequent entity shown in Table 1 As these strains do not contribute directly to the elucidation of the relationship between concomitantly occurring M and NM variants, they are not to be further discussed.

DISCUSSION

Factors contributing to the frequent colonization by mucoid *P aeruginosa* of the respiratory tract in CF patients comprise nosocomial spread, bacterial properties, and the host response to infection.

Our results show that one epidemiological type predominated among the CF patients. Cross-infection may have occurred in several ways

a) All patients visit the same outpatient clinic,
b) During the visits, 10–20 other CF patients are present,
c) At each consultation, all patients are tested by the same respirometric apparatus,
d) At intervals, many of the patients are hospitalized and receive inhalation therapy with moist air Although contaminants have not specifically been isolated from the humidifiers, they have elsewhere been associated with *Pseudomonas* cross-infections,
e) The patients, mostly children, are often in direct contact during play while they are in hospital.

Consequently it may safely be concluded that nosocomial mechanisms have been active in this group of CF patients. Cross-infection may even have been more important in this group than in a group of similar size described by *Diaz et al.* (9) where several serogroups were represented On the other hand, wheth-

of *P aeruginosa* is transmitted as the M or the NM variant, is more difficult to assess. The typing results lend strong support to the supposition that the simultaneously occurring M and NM variants often are derived from one and the same parent strain. In all instances of M NM-variation, the two cell lines were of the same type. Similarly *Diaz et al.* (9) noted that M and NM variants from one and the same specimen invariably were of the same serogroup In both studies, the serogroup persisted for a long time.

The mucoid character is unstable. M NM variation has been observed frequently in both collaborating laboratories. According to our experience (6) the mucoid character is easily lost after storage on nutrient agar slants or stabs and after freeze drying. Rapid freezing to -30° C is often more successful for the preservation of abundant polysaccharide production.

According to *Martin* (21) the mucoid character may be induced by lysogenization. The M-NM-variation would then follow prophage loss. Consequently it is understandable that patients originally infected by an NM strain subsequently may yield mucoid colonies and *vice versa*. Reported experience has been interpreted to indicate that NM colonization frequently precedes the presence of mucoid variants. *Doggett et al.* (11) usually observed NM variants for a while before mucoid colonies emerged. In CF with *P aeruginosa* infection of short duration, or with intermittent episodes of infection, NM strains are more common Mucoid colonies dominate mainly in chronically colonized CF patients (15)

In this connection, it is important to realize that changes in serogroup and phage susceptibility have previously been observed after lysogenization (5) Such changes have been induced both *in vitro* and *in vivo* and are consequently relevant to the present study Similarly pyocine production is governed by episomal DNA (20) This implies that a strain may change from NM to M, simultaneously with a change in epidemiological type. The fact that a predominant number of our M-NM pairs showed identity or near identity

x

tends to render quite strong support to the supposition that the concomitant isolates emerge from the same NM strains.

Since the mucoid character is easily lost upon subculture, it seems as if *in vivo* there exists a continuous pressure by which the selection of M variants is favoured. On the part of the microbe, the capsular substance possessing antiphagocytic properties (22) acts as a virulence factor Host response factors must also be responsible for the high frequency of mucoid strains in CF and bronchiectasis, and not in other chronic pathological states also associated with NM colonization of the respiratory tract such as chronic bronchitis and tracheostoma (6 16) Mucoid strains of *P aeruginosa* are associated with a pronounced and heterogeneous humoral immune response against *Pseudomonas* antigens, whereas the cellular immune response is not increased (9 12, 15 17 18) In CF patients chronically infected with mucoid strains, mucoid substance may either be a poor immunogen or a state of immunological tolerance against mucoid substance may exist. At any rate, neither humoral nor cellular immune response against mucoid substance could be revealed in our CF patients with chronic *P aeruginosa* infection (18) It has therefore been proposed that the immune response in these patients may represent a selective favourization of mucoid strains in the respiratory tract of CF patients (15 17 18)

In conclusion, the typing results show that

a) The co-existing M and NM variants in sputum from CF patients are often of one and the same origin, and
b) True M NM-variation is easily obtained *in vitro*

The M variety may be associated with nosocomial spread, but interference from host factors seem to continuously favour a selection of the unstable M variants. Such mechanisms may at least in part, be responsible for the long period of colonization with mucoid strains of *P aeruginosa* in CF patients.

REFERENCES

1 *Bartell, P F., Orr T F & Chudio B.* Purification and chemical composition of the protective slime antigen of *Pseudomonas aeruginosa.* Infect. Immun. *2:* 543–548, 1970

2. *Bergan T* A new bacteriophage typing set for *Pseudomonas aeruginosa.* 1 Selection procedure. Acta path. microbiol. scand. Sect. B, *80* 177–188, 1972

3 *Bergan T.*, Epidemiological markers for *Pseudomonas aeruginosa.* 1 Serogrouping, pyocine typing—and their interrelations. Acta path. microbiol. scand. Sect. B, *81* 70–80 1973

4 *Bergan T* Epidemiological markers for *Pseudomonas aeruginosa.* 3 Comparison of bacteriophage typing, serogrouping, and pyocine typing on a heterogeneous clinical material. Acta path. microbiol. scand. Sect. B, *80* 91–101 1973

5 *Bergan T & Midtvedt T:* Epidemiological markers for *Pseudomonas aeruginosa.* 4 Change of O-antigen and phage sensitivity after phage infection *in vitro* and *in vivo* of *Pseudomonas aeruginosa.* Acta path. microbiol. scand. Sect. B, *83* 1–9 1975

6. *Bergan, T & Høiby N* Unpublished observations.

7 *Burns M W* Significance of *Pseudomonas aeruginosa* in sputum. Brit. med. J *3* 382–383 1973

8 *Carlson D M & Matthews L. W* Polyuric acids produced by *Pseudomonas aeruginosa.* Biochemistry *5* 2817–2822 1966.

9 *Diaz F Mosovich L. L. & Neter E.* Serogroups of *Pseudomonas aeruginosa* and the immune response of patients with cystic fibrosis. J infect. Dis. *121* 269–274 1970

10. *Doggett R. G* Incidence of mucoid *Pseudomonas aeruginosa* from clinical sources. Appl. Microbiol *18* 936–937 1969

11 *Doggett R G Harrison G M Stillwell, R. N & Wallis E. S* An atypical *Pseudomonas aeruginosa* associated with cystic fibrosis of the pancreas. J Pediat. *68* 215–221 1966

12. *Doggett R. G & Harrison G M Pseudomonas aeruginosa.* Immune status in patients with cystic fibrosis. Infect. Immun. *6* 628–635 1972

13 *Gillies R. R. & Govan J R. W* Typing of *Pseudomonas pyocyanea* by pyocine production. J Path. Bact. *91:* 339–345, 1966.

14 *Høiby N* Epidemiological investigations of the respiratory tract bacteriology in patients with cystic fibrosis. Acta path. microbiol scand. Sect. B, *82* 541–550, 1974.

15 *Høiby N Pseudomonas aeruginosa* infection in cystic fibrosis. Relationship between mucoid strains of *Pseudomonas aeruginosa* and the humoral immune response. Acta path. microbiol. scand. Sect. B, *82* 551 558, 1974

16. *Høiby N* Prevalence of mucoid strains of *Pseudomonas aeruginosa* in bacteriological specimens from patients with cystic fibrosis and patients with other diseases. Acta path. microbiol. scand. Sect. B, *83* 549–552, 1975

17 *Høiby N & Axelsen N H* Identification and quantitation of precipitins against *Pseudomonas aeruginosa* in patients with cystic fibrosis by means of crossed immunoelectrophoresis with intermediate gel. Acta path. microbiol. scand. Sect. B, *81:* 298–308, 1973.

18. *Høiby N Andersen, V & Bendixen, G. Pseudomonas aeruginosa* infection in cystic fibrosis. Humoral and cellular immune responses against *Pseudomonas aeruginosa.* Acta path. microbiol. scand. Sect. C, *83* 459–468, 1975

19 *Jessen O Pseudomonas aeruginosa* and other Green Fluorescent Pseudomonads. A Taxonomic Study Munksgaard. Copenhagen 1965.

20 *Kageyama, M* Genetic mapping of a bacteriocinogenic factor in *Pseudomonas aeruginosa.* II. Mapping of pyocin R2 factor by transduction with phage F116. J gen. appl. Microbiol. *16* 531–535, 1970.

21 *Martin D R* Mucoid variation in *Pseudomonas aeruginosa* induced by the action of phage. J med. Microbiol. *6* 111–118, 1973.

22. *Schwarzmann S & Boring III J R.* Antiphagocytic effect of slime from a mucoid strain of *Pseudomonas aeruginosa.* Infect. Immun. *3*. 762–767 1971

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MULTIPLE FORMS OF STAPHYLOCOCCAL α-TOXIN

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A group of proteins was readily extracted at neutrality from trichloroacetic acid precipitates of staphylococcal culture filtrate supernatants, while α-toxin was dissolved and activated by treating the precipitate with 8 M urea, with acidic buffers or by heating to 90–100 C at neutrality. Heat activation of the precipitate produced a relatively pure α-toxin with a molecular weight of 39,000. α-Toxin was eluted together with three other proteins on hydroxyl apatite chromatography and evidence was obtained for an association between the four proteins. On isoelectric focusing a haemolytic fraction was obtained at pH 6.2, probably due to acid activation of the precipitate formed at the cathodic end of the column. The α haemolytic fractions with pI's of 7.4 and 8.6 were shown to consist of α-toxin only when analysed by acrylamide electrophoresis in the presence of sodium dodecyl sulphate. The haemolytic component with a pI of 9.2 contained two additional components of molecular weights of 7,500 and 18,000. Chromatography of this material on Sephadex G-200 showed that α-toxin and the two proteins appeared as a high molecular complex.

Key words: Staphylococcal α-toxin multiple forms.

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Multiple forms of staphylococcal α-toxin were first demonstrated by *Bernheimer & Schwartz* in 1963 (2) by sucrose density gradient electrophoresis. From electrofocusing studies *Wadström* (14) reported four haemolytic forms. *McNiven et al.* (12) verified these findings and further demonstrated interconversion between two of the forms.

In earlier studies precipitation of supernatants of staphylococcal cultures with trichloroacetic acid (TCA) has yielded an α-toxin in an insoluble form (5). The toxin could be dissolved and reactivated by treatment with urea. In the present work the TCA precipitates have been used in the analysis of the molecular forms of α toxin.

MATERIALS AND METHODS

The Production of Toxin

Staphylococcus aureus strain Wood 46, was propagated as described earlier (6). The medium based on Casamino acids (vitamine-free) was the same as reported previously except that an evaporate of a 50 per cent (v/v) ethanol extract of 10 g yeast autolysate (Oxoid Ltd London) was added per litre medium. Incubation was performed on a rotary shaker (Controlled Environment, Incubator Shaker New Brunswick Scientific Co., New Brunswick, N J USA) 150 strokes/min, for 18 to 22 h. Bacteria were preincubated overnight,

and a 5 per cent inoculum (v/v) in 500 ml medium in 2 litre flasks was used. The incubation was continued overnight and bacteria were then removed by centrifugation and filtration on Millipore filters, pore size 0 45 μ. Proteins from supernatant fluids were precipitated by addition of TCA to a concentration of 5 per cent (w/v) The precipitate was collected by centrifugation, washed twice in 50 per cent ethanol in tris-chloride buffer (0 05 M, pH 7.5) (this buffer was used throughout, when nothing else is mentioned) and then extracted four times with tris-chloride buffer The remaining precipitate, containing most of the α-toxin, was used for further analysis.

Electrofocusing

To electrofocusing columns (LKB Produkter Stockholm) of 110 ml capacity (8101) was added a final concentration of 1 per cent (v/v) carrier ampholyte ULKB Ampholine) in a sucrose gradient with the pH range of 3–10. The anode contained 1 per cent (v/v) sulphuric acid in 60 per cent (w/v) sucrose, and the cathode, which was layered on top of the gradient, consisted of 1 per cent (w/v) sodium hydroxide in distilled water The samples to be analyzed were usually dissolved in 8 M urea. In most cases it was made 0.5 per cent (v/v) with ampholyte, and used as the less dense solution in fractions 12–16 of the total 24 fractions used for preparation of the column. Otherwise the less dense solution contained 0.5 per cent (v/v) of ampholyte, and the dense solution 45 per cent (w/v) sucrose and 1.5 per cent (v/v) ampholyte. Removal of ampholyte and concentration of protein were achieved by precipitation with trichloroacetic acid (TCA) (5 per cent, w/v) The resulting precipitate was washed once with 5 per cent TCA and twice with 95 per cent ethanol.

Protein Determination

This was performed by the Folin-Ciocalteu method (9) using crystalline bovine serum albumin as standard.

Haemolytic Assay

Titration of α-toxin was performed with a 2 per cent suspension of rabbit erythrocytes as described before (6)

Double Diffusion in Gel

A 3 mm thick layer of 1 per cent agarose (Behringwerke Marburg Lahn, W Germany) in tris-chloride buffer was used. Undiluted antiserum and test material were placed at 7 mm distance in holes (3 mm diameter) The antiserum was the same as described earlier and an international standard for staphylococcal anti-toxin, 1 ml containing 20 I U., was obtained from Statens Seruminstitut, Copenhagen (Batch no. 88)

Column Chromatography

Hydroxyl apatite (Bio-Gel HTP Bio-Rad Labs Richmond, Calif., USA) and Sephadex G-200 (Pharmacia AB, Uppsala, Sweden) columns were prepared according to the instructions of the producers. Five grams of dry hydroxyl apatite were applied to the column (diameter 2.5 cm, bed height 3 cm) A Pharmacia column, 45 × 2 5 cm was used for Sephadex G-200, giving a gel volume of 175 ml. Separation was done at room temperature. A peristaltic pump (1200 Varioperpex, LKB was used, and extinction at 280 nm was recorded with a Uvicord 11 (LKB)

Polyacrylamide Gel Electrophoresis

Analytical electrophoresis was carried out in glass tubes (0.5 × 7.5 cm) in a Shandon apparatus (Shandon Scientific Co., London) The same buffer (0.37 M tris-glycine, pH 9.5) was used in the gel and in the electrode vessels (8) When sodium dodecyl sulphate (SDS) was employed, the gels and reservoir buffers were made 0 1 per cent (w/v) with SDS. The samples were dissolved in a small volume of 1 per cent SDS and left at room temperature for at least 1 h before electrophoresis. Immediately before electrophoresis the samples were diluted to an SDS concentration of 0.1 to 0.2 per cent with tris-glycine buffer (0.07 M, pH 9.5) containing 10 per cent glycerol (v/v) and bromphenol blue (0.002 per cent) The separating gel contained 15 per cent (w/v) acrylamide (British Drug Houses Ltd., Poole, England) The gels were polymerized with ammonium persulphate and N,N'-N' tetramethylethylenediamine Staining was accomplished by submerging the gels for 1 h in a solution of 0.2 per cent Coomassie Brilliant blue (R 250) in 50 per cent methanol in water to which 7 per cent of glacial acetic acid was added just before use. Destaining was done with a solution containing 7 per cent acetic acid and 4 per cent methanol. For molecular weight determinations the following marker proteins (Boehringer, Mannheim, W Germany) were run electrophoretically bovine serum albumin molecular weight 67,000 ovalbumin, molecular weight 45,000 bovine pancreatic chymotrypsinogen, molecular weight 25,000 horse myoglobin, molecular weight 17,800 and horse heart cytochrome C, molecular weight 12 400.

RESULTS

The TCA precipitate of supernatants from staphylococcal cultures was partially soluble in the tris-chloride buffer The insoluble frac

and a 5 per cent inoculum (v/v) in 500 ml medium in – litre flasks was used. The incubation was continued overnight and bacteria were then removed by centrifugation and filtration on Millipore filters, pore size 0.45 μ. Proteins from supernatant fluids were precipitated by addition of TCA to a concentration of 5 per cent (w/v). The precipitate was collected by centrifugation, washed twice in 50 per cent ethanol in tris-chloride buffer (0.05 M pH 7.5) (this buffer was used throughout when nothing else is mentioned) and then extracted four times with tris-chloride buffer. The remaining percipitate containing most of the α-toxin, was used for further analysis.

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Column Chromatography

Hydroxyl apatite (Bio-Gel HTP Bio-Rad Labs, Richmond, Calif., USA) and Sephadex G-200 (Pharmacia AB, Uppsala, Sweden) columns were prepared according to the instructions of the producers. Five grams of dry hydroxyl apatite were applied to the column (diameter 2.5 cm, bed height 3 cm). A Pharmacia column, 45 x 2.5 cm, was used for Sephadex G-200, giving a gel volume of 175 ml. Separation was done at room temperature. A peristaltic pump (1200 Varioperpex, LKB) was used, and extinction at 280 nm was recorded with a Uvicord II (LKB).

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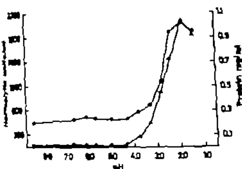


Fig. 1 Effect of pH on α-toxin containing precipitate. Dissolved protein (mg/ml) ● and haemolytic activity ▲

tion contained 60 per cent of the total protein and virtually all of the α-toxin. Haemolytically active α toxin could be prepared from the insoluble precipitate in various ways

Activation of α Toxin by Change in pH

Treatment of the precipitate with alkaline tris-chloride buffers up to pH 9.5 released only an insignificant part of the haemolytic activity The precipitate was partially soluble in NaOH (0.02 M) at room temperature and was completely dissolved by heating at 80° C for 1 min. The solutions were haemolytic and gave a precipitation line against anti-α-toxin on gel diffusion.

When the precipitate was suspended in tris-chloride buffer and the pH lowered by addition of HCl, solution of proteins and release of haemolytic activity took place as shown in Fig 1 On reneutralization a precipitate was formed when the pH reached 4.0. At neutrality a fraction of the α toxin remained in solution in a haemolytically active form, while the rest was precipitated. The ratio of dissolved to precipitated α toxin was dependent on the protein concentration. Increase in protein concentration favoured precipitation of toxin. Storage of the dissolved toxin resulted in inactivation and precipitation, the half-life of haemolytic activity being 2–3 h at room temperature

Precipitates dissolved in glycine-HCl buffer (pH 2.6 0.05 M) were run on Sephadex G-200 columns equilibrated with the same buffer Fractions were tested for haemolytic activity and precipitinogenic properties and analyzed by electrophoresis in the SDS system. A portion of the α toxin was eluted in the void volume in a low-haemolytic form along with other proteins of various molecular sizes, probably indicating the presence of a complex. Active α toxin was eluted in a broad zone the peak activity having a Kd corresponding to a molecular weight of about 40,000 In a similar but more acidic system (HCl/0 02 M) relatively less low-haemolytic α toxin could be demonstrated in the void volume, and a correspondingly greater part as active toxin.

Activation of α Toxin by Urea

The α toxin containing precipitate was readily soluble in 8 M urea (in tris-chloride buffer) When the protein content was less than 1 mg/ml a 20 fold dilution of the solved protein in tris-chloride buffer (of the urea-solved protein) gave no visible precipitation at room temperature. Heating to 60 C gave immediate precipitation of the proteins. With a protein content of 5 mg/ml a precipitate was formed on diluting with tris-chloride buffer to a urea concentration of 3 M Analysis of this precipitate in the SDS electrophoresis system showed that α-toxin was the dominating component. The haemolytic titre of toxin dissolved in 8 M urea remained un-

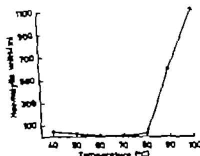


Fig 2 Activation by heat of α-toxin from TCA precipitates. Suspensions in tris-chloride buffer (pH 7.5) were heated for 2 min. Haemolysis was measured in supernatants after centrifugation.

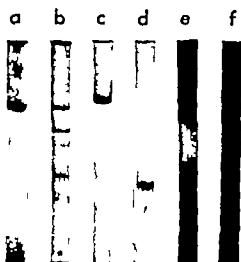


Fig 3 Sodium dodecyl sulphate-polyacrylamide electrophoresis. (a) Heat activated staphylococcal α toxin (b) Eluate from hydroxyl apatite (0 1 M phosphate) (c) Insoluble fraction at neutrality of b after precipitation with trichloroacetic acid (d) Soluble fraction of b at neutrality (e) Isoelectric focusing of b, fraction with pI 9.5 (f) Isoelectric focusing of b, fraction with pI 8.4

changed for several days at room temperature Gel filtration on Sepharose 4 B equilibrated with 8 M urea in tris-chloride buffer showed as in the acidic system that a minor fraction of α toxin was eluted as a complex in the void volume whereas the haemolytically active form was eluted as a broad single peak.

Activation of α Toxin by Heat

Suspension of the α toxin containing precipitate in tris-chloride buffer gave only a small release of active toxin The low haemolytic activity disappeared entirely by heating at 60 70 C for ? min while a considerable reactivation was observed by heating at 90 100 C (Fig ?) Polyacrylamide gel electrophoresis in the SDS system of supernatants after centrifugation of heat reactivated material showed that mainly α toxin was released from the complex precipitate (Fig 3a) α Toxin which was activated by heating behaved in the usual manner with a rapid decline in haemolytic activity on storage and formation of a precipitate.

Partial Purification of α Toxin on Hydroxyl Apatite Columns

The precipitate was dissolved in tris chloride buffer containing 8 M urea and applied to a hydroxyl apatite column equilibrated with the same buffer The column was then washed with the buffer followed by a rinse in distilled water The proteins passing through the column (Fig 4 peak I) were examined in the SDS system and shown to be of low molecular weight (MW<15,000) Stepwise elution was then performed with phosphate buffer pH 7.0 At a concentration of 0.05 M (Fig 4 peak II) four non-haemolytic proteins were found. α Toxin was eluted in a broad zone with 0 075 M phosphate buffer and as a narrow peak with 0.1 M (Fig 4 peak III) Three additional major proteins with molecular weights of 27,500, 18 000 and 12 000 were eluted at a concentration of 0 1 M (Fig 3b) An increase in phosphate concentration to 0.3 M led to elution of only small amounts of additional

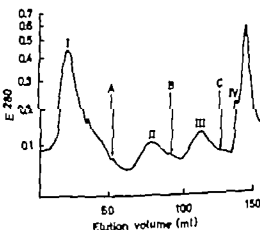


Fig 4 Elution diagram of crude TCA precipitated α-toxin applied in 8 M urea on a hydroxyl apatite column. Extinction at 280 nm. Stepwise elution with phosphate buffer pH 7.0.

A. 0 05 M phosphate
B. 0.1 M phosphate
C. 0.1 M phosphate + 8 M urea.

proteins. However elution with 0 1 M phosphate buffer in 8 M urea showed that a considerable amount of protein was retained by the column (Fig. 4 peak IV and peak V) Fractions from peak V showed haemolytic activity with maximal titres corresponding to the descending part of the curve. Disc electrophoresis revealed the same four major proteins as seen in peak III one of them being α-toxin. Dilution of the peak fraction of V with distilled water to a urea concentration of 1 M resulted in precipitation. The precipitate and the haemolytic supernatant had identical composition when examined in the SDS system.

The findings indicated a possible interaction between α-toxin and the three other proteins. To examine this further fractions from peak III (0 1 M phosphate) were precipitated with TCA and washed with tris-ethanol in the usual manner The precipitate was readily dissolved in 0 02 M HCl. When chromatographed on Sephadex G-200 equilibrated with 0.02 M HCl, the four components separated according to their molecular weights indicating dissociation of the components under these conditions.

Extraction of the precipitate with a tris-chloride buffer (pH 8.5 0.05 M) failed to dissolve the α toxin, most of the three other components being extracted (Fig 3 a, d) Chromatography of the tris-soluble fraction on Sephadex G-200 (tris-chloride, pH 8.5 0.05 M) showed that the three components were eluted in a single peak, indicating that they existed as a complex.

The residuum after extraction with tris-chloride containing mainly α-toxin, was dissolved in a few drops of 8 M urea and subjected to chromatography under the same conditions. The bulk of toxin was eluted in the void volume in a low-haemolytic form. The void volume also contained what remained of the components of MW 27,500, 18,000 and 12,000 after tris extraction.

The experiments showed that the three non-haemolytic components existed as a soluble complex at neutrality In the presence of free α-toxin a higher molecular complex was

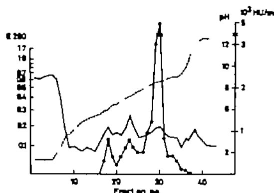


Fig 5 Electrofocusing of crude TCA-precipitated α-toxin in the presence of 6 M urea in a pH 3–10 gradient. ---- pH, —— extinction at 280 nm, ●——● haemolytic activity The sample was applied in the light solution.

formed which tended to precipitate at neutrality

Electrofocusing Experiments

When tris-buffer extracted TCA-precipitates of supernatants were dissolved in 8 M urea and distributed in the low density solution, a faint precipitate was formed in the column. During runs of 48 h the precipitate accumulated in the acidic part of the column (pH 2.0–6.0) Analysis of the precipitate in the SDS system showed a protein composition analogous to that of the sample applied. Haemolytic peaks were found at pH 6.2, 7 4 8.6 and 9.2, the peak at 8.6 showing the highest titre, which was in agreement with the findings of *McNiven et al.* (12) When identical samples were electrofocused in 6 M urea, no immediate precipitation took place During the first 24 h, however a precipitation ring was formed at the anodic end slowly moving to the acidic end during the ensuing 24 h. Higher maximal activities were obtained with 6 M urea, but with haemolytic peaks in the same pH regions (Fig 5)

α Toxin with a pI of 6.2

The finding that the protein pattern of the precipitate formed in the acidic part of the column was identical to that of the haemolytic fraction at pH 6.2 suggested that this

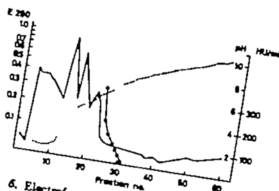


Fig. 6. Electrofocusing of crude TCA-precipitated toxin in a pH 3–10 gradient. --- pH —— extinction at 280 nm, ●——● haemolytic activity. The sample was dissolved in 8 M urea and applied in the light solution at the cathodic end of the column (fractions 5–8 of 24).

haemolytic fraction was formed by solution of the precipitate. When the sample was applied at the cathodic end of the column in fractions 5–8 (of a total of 24) only the haemolytic peak at pH 6.2 was found (Fig. 6). A similar result was obtained when α toxin, partially purified on hydroxyl apatite, was used as the sample. These findings suggested that a precipitate containing α toxin was formed which moved due to gravity to the acidic region of the column, where it dissociated and released free α toxin.

α Toxin with a pI of 7.4 and 8.6

Isoelectric focusing of α-toxin partially purified on hydroxyl apatite and applied in the light solution of the anodic half of the column showed a maximal activity at pH 8.6. A haemolytic peak or shoulder was found at pH 7.4. Haemolytic activity was found and disc electrophoresis showed the presence of α toxin in all fractions from pH 7.4 to 8.6. Its molecular weight was 39,000. There was no evidence of a peptide ligand at pH 7.4.

α Toxin with a pI of 9.1

Electrofocusing of material eluted by 0.1 PO from the hydroxyl apatite column showed haemolytic activity in the fractions from pH 9.0–9.3. Analysis of this haemolytic fraction in the SDS system showed the presence of α toxin and proteins of MW 27,500 and 18,000. Relatively less of the component of MW 12,000 was found. This finding might indicate a protein complex or a coinciding pI of non associated proteins. This was examined by electrofocusing the tris-chloride extract of the 0.1 M phosphate fraction from hydroxyl apatite, which contained very little α toxin. The protein of MW 12,000 was found in the strongly alkaline part of the column (pH>11.5) and its pI could not be determined. The protein of MW 18,000 had an pI of 9.5 (Fig. 3 e). A peak at pH 8.4 contained the proteins with MW 27,500 and 18,000 which indicated that the two proteins were associated (Fig. 3 f). No protein was found at pH 9.1 supporting the assumption of a complex of α-toxin and the two proteins with this pI.

DISCUSSION

Analysis by ultracentrifugation has shown that α toxin appears as a haemolytic component with a sedimentation coefficient of 3S, as a non-haemolytic component with a coefficient of 12S and as an insoluble aggregate (2). It has also been shown that the 3S component can be further disaggregated to a 1.9S component in 8 M urea (1). Molecular weight determinations by various methods have indicated that the 3S component consisted of a mixture of molecules of weights between 21 000 and 50 000 (4). The finding of several haemolytic fractions on electrofocusing is further evidence for the heterogeneous nature of α toxin.

The molecular associations of α toxin seem to be elucidated by the effect of heat, which has been repeatedly studied for the last 70 years. Rapid inactivation of haemolytic activity at 60 C has been a common finding, while reactivation of toxin at higher temperatures has not been consistently reproduced. *Tager* (13) demonstrated that a variety of autolytic products from bacteria, and also other substances such as pepsin and lecithin, combined with the toxin at 60 C, but not at 80 C. *Manohar et al.* (11) showed that the characteristic biological effects of

α-toxin reappeared on reheating the 60° C inactivated crude toxin to 100° C. *Cooper et al.* (3) interpreted the reactivation by heating as a release of trapped, soluble toxin, while *Arbuthnott et al.* (1) explained the phenomenon as partial reversal of the aggregation at elevated temperatures. Under the present experimental conditions there was a definite reactivation of toxin by short heating to 90–100° C. Furthermore, nearly pure α-toxin was released from the complex precipitate, showing that α toxin is a rather stable protein. It would seem reasonable to regard the heat reactions of α toxin as a consequence of its tendency to form apolar bonds. The strength of apolar bonds increases in the region of 0–60° C (10). A further increase in temperature may release heat stable proteins. The finding by *Tager* that lecithin was bound at 60° C, but not at 80° C, is of special relevance in this connection.

The difference between the insoluble aggregate and the non-haemolytic 12S component from purified preparations of α toxin would then be one of random or ordered arrangements of toxin subunits. Proteins liable to form apolar bonds would be bound to α-toxin. The crude TCA precipitates from supernatants contained a group of proteins which was readily extracted at neutrality. Systematic analysis of these proteins has not yet been undertaken, but at least fibrinolysin was completely extracted at neutrality which makes the trapping of proteins in the aggregate unlikely. The insoluble fraction contained a number of proteins of varying size. Even extraction with 4 M urea at neutrality removed proteins of low molecular weights only indicating stable bonds. The four proteins eluted from hydroxyl apatite with 0.05 M phosphate and the three proteins of MW 27,500, 18,000 and 12,000 were all readily soluble at neutrality when α-toxin was not present, demonstrating the importance of the toxin molecule in the formation of the aggregate. The binding of toxin to lipids (7) and the activating effect of urea and acidic or basic conditions, also supported the importance of apolar bond formation in the reactions of α toxin.

The finding on electrofocusing of pure α-toxin in fractions of pH 7.4 and 8.6 indicated at least that no peptide ligand caused the difference in pI. The fact that α toxin was found in all fractions between pH 7.4 and 8.6 indicated interconversion between the two forms.

The formation of a precipitate at the acidic end of the electrofocusing column has been noted repeatedly (1, 12, 14). Dissociation of the precipitate and release of active α toxin would seem to explain adequately the α toxin peak at pH 6.2. α Toxin was always found in the precipitate and could be activated by the usual means. This may explain the findings by *Wadström* (14) of an α haemolytic fraction with pI 4.5–5.5.

Conditions were not ideal for the analysis of the haemolysin with the pI 9.1 since low concentrations of protein were found. This was expected from the finding by *McNiven et al.* (12) that treatment with urea partially converted the toxin of pI 9.1 to the pI 8.6 form. The pattern of 4 proteins at this pI was repeatedly found, suggesting an association between them. The two low molecular components had a pI above 9.1 which may explain why the complex had a higher pI than that of free α toxin.

REFERENCES

1. *Arbuthnott J. P., Freer J. H. & Bernheimer A. W.:* Physical states of staphylococcal α-toxin. J. Bact. *94*: 1170–1177, 1967.
2. *Bernheimer A. W. & Schwartz L. L.:* Isolation and composition of staphylococcal α-toxin. J. gen. Microbiol. *30*: 455–468, 1963.
3. *Cooper L. Z., Madoff M. A. & Weinstein L.:* Heat stability and species range of purified staphylococcal α-toxin. J. Bact. *91*: 1686–1692, 1966.
4. *Coulter J. R.:* Production, purification, and composition of staphylococcal α-toxin. J. Bact. *92*: 1655–1662, 1966.
5. *Dalen A. B.:* The induction of staphylococcal α-toxin by histidine. J. gen. Microbiol. *74*: 61–69, 1973.
6. *Dalen A. B.:* Production of staphylococcal α-toxin in a defined medium and identifica-

tion of a stimulating factor from yeast extract. J gen. Microbiol 74 53–60 1973
7 F eer J H., A buthnott J P & Bernheimer A W Interaction of staphylococcal α-toxin with artificial and natural membranes. J Bact. 95 1153–1168, 1968.
8 Hjertén S., Jerstedt S & Tiselius A Some aspects of the use of continuous and discontinuous" buffer systems in polyacrylamide gel electrophoresis. Analyt. Biochem. 11 219–223
9 Lowry O H R seb ough N J., Farr A L. & Randall R J Protein measurement with the Folin phenol reagent. J biol. Chem. 193 265–275 1951
10 Mahler H R. & Cordes E. H Biological Chemistry Harper & Row Ltd London, 1967
11 Manohar M., Kumar S & Lindorfer R. K. Heat reactivation of the α-hemolytic dermonecrotic, and lethal activities of crude and purified staphylococcal α-toxin. J Bact. 91 1681–1685.
12 McNiven A C Owen P & Arbuthnott, J P Multiple forms of staphylococcal alpha-toxin. J med. Microbiol. 5 113–122, 1972.
13 Tager M. Factors modifying the reaction of alpha staphylolysin to heat. Yale J. Biol. Med. 14 69–88, 1941
14 Wadström T Studies on the extracellular proteins from Staphylococcus aureus. IV Separation of α-toxin by isoelectric focusing. Biochim. biophys. Acta (Amst.) 168 228–242 1968.

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GAS CHROMATOGRAPHY OF BACTERIAL WHOLE CELL METHANOLYSATES

VII Fatty Acid Composition of Acinetobacter *in Relation to the Taxonomy of* Neisseriaceae

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The cellular fatty acids of seventeen *Acinetobacter* strains were determined. Most acids identified were previously found in neisseriae and moraxellae Specific for *Acinetobacter* was 2-hydroxydodecanoic acid and a few minor unidentified components. The fatty acid data were analysed by numerical methods and compared with previous results obtained for neisseriae and moraxellae. The findings were consistent with genetic evidence for some affinities of genus *Acinetobacter* to genus *Moraxella* and "false neisseriae Occasionally a high resemblance in fatty acid pattern was demonstrated between *Moraxella* strain and certain strains of *Acinetobacter* and also between an *Acinetobacter* strain and certain "true neisseriae" Still, the acinetobacters constituted one single cluster separated from the other genera of *Neisseriaceae*.

Key words *Acinetobacter* *Neisseriaceae* fatty acid composition taxonomy

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The taxonomy of *Neisseriaceae* has been extensively studied and particular genetic relationships between species of the oxidase positive genera *Neisseria* and *Moraxella* have become evident and widely accepted (7 17) As regards the oxidase negative genus of the family *Acinetobacter* corresponding knowledge is more limited. The question of a subdivision of *Acinetobacter* into species is presently unsolved, and the relatedness of this genus to neisseriae and moraxellae has not been fully elucidated (7 17)

Grouping of *Neisseria* and *Moraxella* strains based on fatty acid composition (20) generally confirmed the classification derived from genetic analyses. The high degree of consistency suggested that a similar investigation of representative strains of *Acinetobacter* might add significant information with regard to their classification. Furthermore, gas chromatographic analysis might contribute to the identification of these bacteria.

This communication presents the fatty acid composition of representative *Acinetobacter* strains and compares the results with our previous findings within *Neisseria* and *Moraxella* (20)

TABLE 1 *Some Characteristics of the Acinetobacter Strains Examined* [1]

Identification number	Previous name [a]	Collection strain number ATCC	Collection strain number Other [b]	Per cent guanine + cytosine of DNA		DNA homology group	Phenotypic group	Acid production from glucose	Haemolysis/gelatin liquefaction	Growth with citrate as source
1	*Acinetobacter anitratus*	17905		41 [d]		1	A1[f]	+	—	+
2	*Moraxella glucidolyt ca*	17978		42		1	A1	+	—	+
3	"Vibrio 01"	11171		40		1	A2	—	—	w
4	*Mima lwoffi*	17959		42		1	A3	—	—	+
5	*Bacterium anitratum*	15149		43		1		+	—	+
6	*Achromobacter haemolyticus*	17906		45		2	B4	+	+	+
7	*Moraxella lwoffi*	17977		45		2		—	+	+
8.	*Achromobacter anitratus*†		8		39.5 [e]			+	—	+
9.	*A hromobacter anitratus* †		9		39.5		B4	+	+	+
10.	*Achromobacter lwoffi* †		881/57		41.5		B2	—	—	+
11	*Achromobacter haemolyticus* †		2408/57		41.5			+	+	+
12	*Alcaligenes haemolysans*	17988	4233/62	44	41.0	3	B3	—	+	+
13	*Achromobacter metalcaligenes*	17909		44		4	B1	—	—	+/
14	*Mora ella lwoff*	17985		46	43.0	5	B2*	—	—	w
15	*Achrom bacter citroalcaligenes*	17908		42		6	B1	—	—	+
16.	*Achromobacter anitrata*	17924		45		6		+	—	+
17	*Acinetobacter calcoaceticus* ✤		BD4		41.5			+	—	+

All strains except identification nos. 8, 9 10 and 11 have been examined in genetic transformation by *Juni* (25) and found compatible with *Acinetobacter calcoaceticus* BD4

[a] Name when ntered in The American Type Culture Collection (ATCC) (16, 34) or used in publications indicated in the table.

† Nomenclature of *Baur* (8) ✤ Nomenclature of *Juni (25)*

See refs. 8 and 25 (strain BD4)

The figures in this column refer to determination by thermal melting point (23)

[e] The figures in this column refer to determination by buoyant density (9) strain BD4 examined by *M Fiandt* and *K. Be e* using the same method (unpublished)

Homology expressed in nucleic acid hybridization (23)

Grouping by numerical analysis of nutritional and physiological properties (3)

only partially characterized strains (3)

[1] Compiled from investigations in several laboratories (refs. 3 8 34 unpublished report by the International Committee on Systematic Bacteriology Subcommittee on *Moraxella* and Allied Bacteria, 1970 and own in estigations by methods as in ref 8)

+/ — positive and negative results recorded.

w weak growth recorded in some laboratories.

MATERIALS AND METHODS

Bacterial Strains

The seventeen strain of I t bacte examined (Table 1) all belong t the ge as currently defined (7 17) They er el as to represent the wide range denced by DNA base composition, l dization, and cultural-biochem 3 9 13 23 25 39) The st and moraxellae included for een described and examined pre

Growth and Harvesting

The bacteria were cultivated in a humid atmosphere on blood agar plates at 35 C for 20 hours and harvested as detailed previously (21) All strains were cultivated on one and the same batch of medium and processed on two successive days.

Chemicals and Chemical Procedures

Solvents were of pro analysis grade and redistilled before use Fatty acid methyl ester stan-

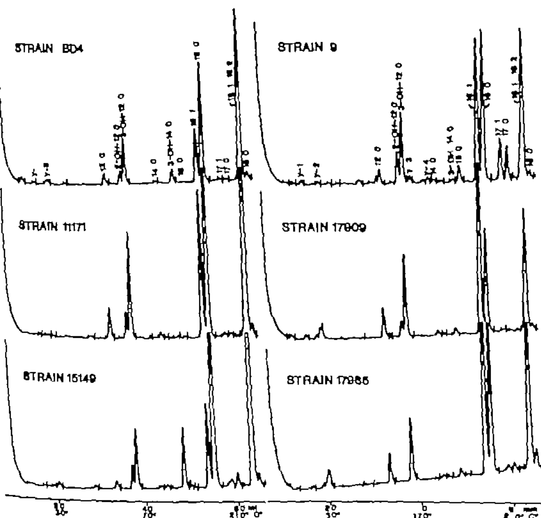


Fig. 1 Representative fatty acid profiles of Acinetobacter *strains.* Column 10 per cent W982 on Gas-Chrom Q, 200 × 0.2 cm (glass) Temperature programmed 2 C/min from 100 C to 250° C. Flow rate of carrier gas 30 ml/min. Further chromatographic details are given in ref 19 the chromatograms presented correspond to profil 2 of the flow scheme of that reference. Key to the fatty acid symbols is indicated in Table 2.

dards were purchased from Applied Science Lab. Inc. and Supelco Inc.

The chemical procedures hav been described previously (19) Lyophilized bacterial cells (1–10 mg) were depolymerized by 2 N HCl n anhydrous methanol. After centrifugation, the supernatants were derivatized by trifluoroacetic anhydride and the fatty acid methyl esters selectively extracted by hexane

Fatty Acid Analysis and Peak Identification

The f tty acid methyl esters were analysed on Hewlett-Packard 5750 gas chromatograph as detailed previously (19 20) Two glass columns (200 × 0.2 cm) were used, packed with either 10 per cent WR82 or EGA on Gas-Chrom Q (100–120 mesh) The flow rate of carrier gas (N) was 30 ml/min. The temperature programme usually started at 120 C and was increased by 2 C per min up to 250° C. Peak areas were calculated by an electronic integrator and given as percentages of the total fatty acids (20)

For peak identification, a sequence of several methods was employed (19, 20) Primary characterization was done by comparing the retention times of unknown peaks to those of fatty acid methyl ester standards. The linear relationship

TABLE 2. *Cellular Fatty Acids of Acinetobacter Strains*[1]

Identification number	Collection strain number	y1	y2	12:0	2-OH 12:0	3-OH-12:0	y3	y4	14:0	3-OH-14:0	15:0	16:1	16:0	y5	17:1	17:0	x-6	18:2	18:1	18:0	others
		Fatty acid																			
1	ATCC 17903	0.1	1.0	3.0	2.2	6.2	–	0.5	0.4	3.6	0.7	12.3	21.7	0.5	3.9	3.2	–	tr	37.8	1.2	1.4
2.	ATCC 179[illegible]	0.3	1.4	3.7	3.7	9.8	–	0.9	0.4	7.9	0.5	11.6	20.8	tr	2.5	1.9	–	tr	32.6	1.2	0.7
3	ATCC 11171	0.1	0.1	3.2	3.0	12.8	–	–	0.5	0.3	0.2	15.7	23.4	0.2	0.3	0.3	0.2	3.4	33.0	1.3	1.3
4	ATCC 17959	0.2	0.4	2.9	2.8	8.9	–	0.4	0.5	5.7	0.4	22.1	29.7	tr	2.3	0.9	0.3	0.6	24.5	tr	0.7
5	ATCC 15149	tr	0.5	0.7	2.8	7.0	–	0.3	0.3	6.8	0.2	9.1	23.1	–	0.9	1.4	–	tr	42.4	1.5	1.6
6	ATCC 17906	–	[illegible]	3.0	3.0	11.3	–	–	0.2	0.3	0.6	11.8	22.0	–	2.2	2.8	–	5.3	34.3	1.4	1.1
7	ATCC 17977	0.3	0.8	2.8	4.7	10.4	–	0.3	–	–	0.3	11.6	23.2	tr	3.8	4.6	–	0.7	34.8	1.7	0.6
8.	8	0.1	0.9	3.0	0.5	7.4	–	0.5	0.3	3.7	1.2	11.7	24.2	0.5	2.8	2.5	–	tr	38.3	1.2	0.9
9	9	0.4	0.4	1.8	4.9	11.3	0.5	0.7	0.2	0.4	2.1	19.8	20.4	0.3	3.3	4.3	0.3	4.3	21.0	0.6	1.9
10.	881/57	0.2	0.6	2.5	1.7	9.8	–	–	0.5	0.8	tr	22.7	19.6	tr	0.4	0.4	0.3	1.1	36.4	2.1	0.8
11	2408/57	0.2	0.3	2.1	3.8	12.5	–	–	0.5	0.4	tr	14.8	19.4	tr	0.4	0.4	0.2	2.4	35.9	2.4	2.0
12.	ATCC 17988	0.2	0.3	1.4	4.6	10.6	–	0.2	0.3	0.3	0.7	17.6	16.7	2.4	2.4	4.4	0.1	1.3	33.7	1.2	1.9
13	ATCC 17909	0.4	2.8	4.3	2.4	15.1	–	–	0.5	1.1	–	34.3	17.0	tr	0.2	tr	0.4	tr	19.9	1.3	0.2
14.	ATCC 17985	0.3	3.2	3.9	–	8.0	–	0.1	0.4	1.0	0.2	30.2	24.2	tr	0.6	0.8	0.3	1.0	22.8	2.5	0.6
15.	ATCC 17908	0.1	0.3	1.1	5.1	11.0	–	–	0.5	0.9	2.2	21.9	11.3	–	5.9	7.3	0.4	0.6	27.7	1.2	1.5
16.	ATCC 17924	0.5	0.2	6.6	7.4	14.7	–	–	0.9	0.8	–	15.6	21.3	–	0.6	1.1	–	4.7	25.7	2.2	0.5
17	BD4	0.3	1.2	1.5	2.9	10.6	–	–	0.3	2.6	–	11.0	24.5	tr	0.1	0.3	–	3.2	37.1	1.9	1.0

[1] See text and refs. 19 and 20 for experimental details.

See text and Table 1 for complete strain designation.

Key to the fatty acid designation: the figure before colon indicates the number of carbon atoms in the chain, the one after colon denotes the number of double bonds (the position of the double bonds has not been determined); the symbol *OH* indicates a hydroxy group; its position relative to the carboxyl group is denoted by the figures 2 and 3 respectively. y and denote unidentified components. The x-6 component corresponds to the compound of identical designation in neisseriae and moraxellae (20). The names of all fatty acids except OH-12:0 (2 hydroxy-dodecanoic acid) were given in a previous report (20).

As previously (20), "others" denotes components eluting after C_{18} (up to C_{22}) fatty acids.

The fatty acid concentrations are given as percentage of the total amount. The symbol "tr" (trace) indicates that the acid contributes less than 0.1 per cent of the total.

between chain length among homologous fatty acid methyl esters and the logarithm to their retention times was also utilized. Further characteristics were obtained by determination of partition parameters in acetonitrile/hexane and by reduction. Tentative identification was confirmed by mass spectrometric analysis.

All strains were analysed for cyclopropane fatty acids after alkaline methanolysis by the procedure of Brian & *Gardner* (5)

Procedures of Numerical Analysis

The similarity between the fatty acid patterns of all strains was analysed by the numerical procedure described earlier (4 18 20) using the transformation formula $y = ln (x + 1)$ where x is the relative quantity of each fatty acid as presented in Table 2. To allow comparison with previous data on neisseriae and moraxellae (20) quantities below 0.5 per cent of total (previously designated as trace quantities) were all substituted by the value 0.2 as in the earlier study (20)

Cluster analysis was carried out by the unweighted pair group method (36) and by the procedure of principal components analysis (2)

RESULTS

Fatty Acid Composition

The fatty acid composition of the strains of *Acinetobacter* is shown in Table 2, and six examples of "fatty acid" profiles are given in Fig 1 In general, the fatty acid patterns of the selected strains are fairly similar All are characterized by high levels of 3–OH–12 0* 16 1, 16 0 and 18 1 The 12 0 17 1 17 0 and 18 2 fatty acids are observed in all strains, but in lower concentrations

Only a few differences in fatty acid composition between the *Acinetobacter* strains appear significant. Most striking is the lack of 2-OH–12·0 only in strain ATCC 17985 The two C_{14} acids, 14 0 and 3–OH–14 0 are encountered in all strains but ATCC 17977 Notable is also the presence of an unidentified component, y-3 exclusively in strain no 9

The fatty acid pattern of *Acinetobacter* is comparable to that found in neisseriae and moraxellae (20) Thus the ratios between C_{18} and C_{16} acids are all within the range 0.8–2.2 and the ratios between saturated and unsaturated acids are within the range 0 4–0 7 Being intermediate between "true" and "false neisseriae" these values partly overlap with the values for "classical moraxellae" and *Moraxella phenylpyrouvica* (ref. 20 Table 2)

The fatty acid 2–OH–12 0 and the unidentified components labelled *y* (Table 2) were not found in neisseriae and moraxellae (20) In contrast to the y components, the identity of 2–OH–12·0 was well established. Both the GLC retention characteristics and the p-value in acetonitrile/hexane (19) were similar to that of the commercial standard. In addition the mass spectrometric analysis also confirmed the identity the fragmentation pattern was indistinguishable from that of the reference and both the molecular ion (m/e 230) and the characteristic fragment at m/e 90 (33) could be detected. The compounds y-1 y-2, and y-3 are relatively polar as judged by p-value determinations, but due to their small amounts and heterogeneity the mass spectrometric analyses were inconclusive.

Cyclopropane fatty acids are commonly encountered in bacteria (3.) and cis-9 10-methylene–hexadecanoic acid has previously been reported as a major fatty acid of an *Achromobacter* strain (22) In the presently examined strains, cyclopropane fatty acids could not be detected.

Numerical Analysis

The fatty acid composition of the individual *Acinetobacter* strains (Table 2) was compared and also related to that of the previously analysed neisseriae and moraxellae (20) The similarity matrix appears in Fig. 2 and its upper section shows the intrageneric indices for *Acinetobacter* For the sake of clarity the sequence of the strains in the similarity matrix (Fig. 2) has been arranged in the same order as in the phenogram (Fig. 3 see below)

In Fig. 2, relatively high intra-*Acinetobac*

* Key to the fatty acid designation the figure before colon indicates number of carbon atoms in the fatty acid chain, the one after the colon denotes number of double bonds. For further details see footnote to Table 2.

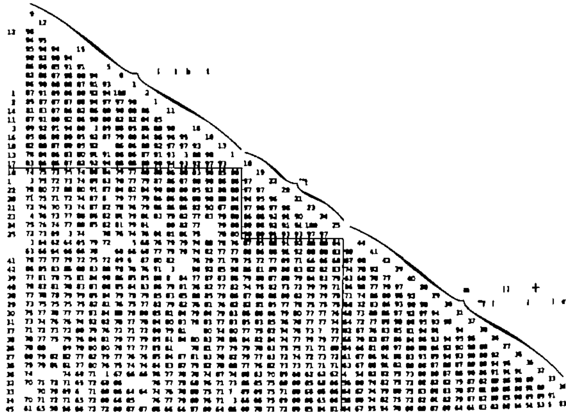


Fig 2 Similarity matrix of Neisseriaceae strains based on their fatty acid composition. Key to the strain identification numbers nos. 1–17 include the *Acinetobacter* strains as specified in Table 1 and in the same order of succession as in Fig. 3 nos. 18–45 are oxidase positive strains previously examined (11 14 20) no. 18 is *N gonorrhoeae* 21319/70 19 *N gonorrhoeae* 562/71 20 *N meningitidis* M1 21 *N meningitidis* B8152/66 22 *N cinerea* 159/62, 23 *N flavescens* ATCC 13120 24 *N flavescens* NCTC 8263 25 *N elongata* M2, 26 *N catarrhalis* No 11 27 *N catarrhalis* 13074/62 28 *N ovis* 199/55, 29 *N ovis* 37/59 30 *N caviae* ATCC 14659 31 *N caviae* NCTC 10293 32 *M nonliquefaciens* 4663/62 33 *M nonliquefaciens* NCTC 7784 N-a, 34 *M nonliquefaciens* 3067/66 N-a, 35 *M bovis* ATCC 10900 N-a, 36 *M bovis* 4 N-b 37 *M lacunata* ATCC 17967 38 *M lacunata* NCTC 7911 39 *M osloensis* A1920, 40 *M osloensis* 5873 41 *M phenylpyruvica* 2863 42 *M phenylpyruvica* ATCC 17958, 43 *M kingae* 4177/66 N-a, 44 *M kingae* 9076/70 N-a, 45 *M urethralis* WM16.
See text and ref 20 for definition and explanation. The differences between similarity indices of this matrix and the corresponding indices of ref. 20 Table 3 are due to the increased number of fatty acids computed in the present study

ter indices are seen (all values except two are above 80) The high intergeneric indices between some *Acinetobacter* strains and *Neisseria meningitidis N gonorrhoeae and N cinerea* are also notable. Evident from this figure is also the high similarity between *M phenylpyruvica* ATCC 17958 and some acinetobacters, and the low affinity of the strain of *M urethralis* (tentatively named (27)) to all strains of *Acinetobacter*

The strains classified as *Acinetobacter* render a distinct phenogram cluster clearly separated from those of neisseriae and moraxellae (Fig 3) This distinction is also brought out by the principal components cluster analysis seen in Fig 4

The homogeneity of *Acinetobacter* in terms of fatty acid composition is indicated by the figure 88.9 ± 4.5 in Table 3 This table also points out the similarities between *Acinetobacter* and the other previously analysed groups of *Neisseriaceae*

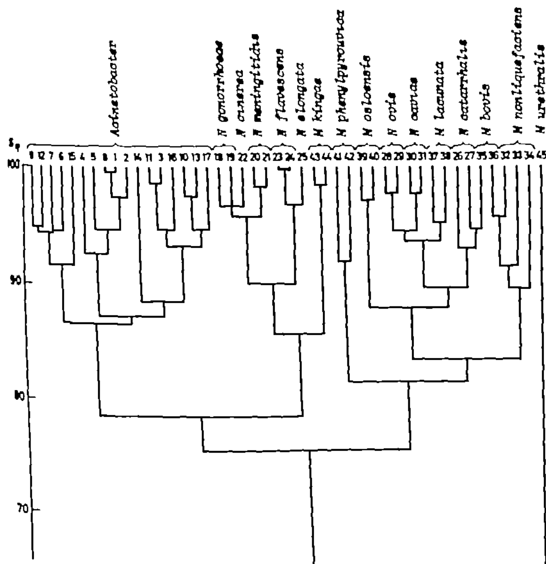


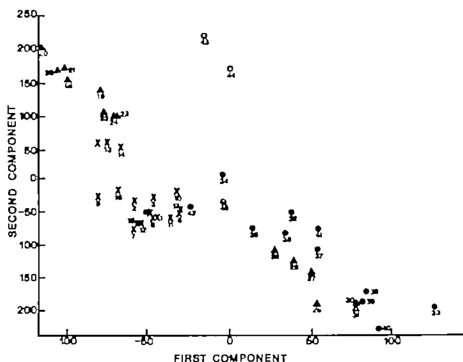
Fig. 3. *Phenogram of strains of the* Neisseriaceae *based on fatty acid composition.* The phenogram was obtained by unweighted pair group analysis (36) of the similarity matrix (Fig. 2).

DISCUSSION

The oxidase negative non-motile Gram-negative coccobacilli previously placed in such taxa as *Achromobacter*, *Alcaligenes*, *Mima*, *Herellea*, *Bacterium anitratum* and *Moraxella lwoffi* were referred to the genus *Acinetobacter* by *Brisou & Prévot* (6). This unification has recently been supported by genetic (23, 25) and phenotypic (3, 24) evidence. However the degree of intrageneric homogeneity is presently unclear. Proposals exist both for a subdivision of the genus (3, 30, 32) and for including the whole group in one single species, *Acinetobacter calcoaceticus* (7, 17, 28).

The degree of homogeneity in fatty acid composition encountered among the *Acinetobacter* strains is expressed by an intragroup similarity index of 88.9 (Table 3). In comparison, the group of "true neisseriae" ex-

37*



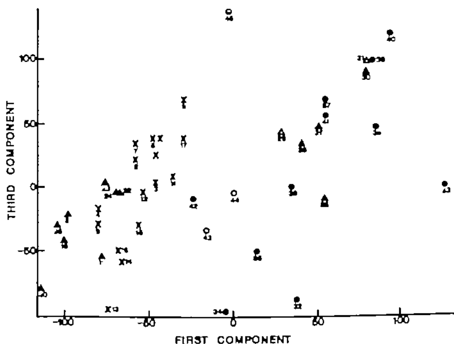


Fig 4 Two-dimensional representation of similarities between Neisseriaceae strains based on principal components analysis (2) of fatty acid composition.
Key to strain identification numbers are given in Table 1 and Fig. 2.
▲ "true neisseriae △ "false neisseriae ● *Moraxella* ○ *M. kingae* ◒ *M. urethralis*
x *Acinetobacter*

TABLE 3 *Inter- and Intragroup Similarity Indices*[1] *of* Acinetobacter *and other Taxonomic Groups within* Neisseriaceae

Group[2]	Variable[3]	Intergroup similarity index (vs. *Acinetobacter*)	Intragroup similarity Index[4]
Acinetobacter	I_φ	–	88.9
	Δ	–	3.7
	σ	–	4.5
True neisseriae (group I)	I_φ	80.6	93.3
	Δ	4.6	3.2
	σ	5.5	3.7
False neisseriae (group II)	I_φ	79.8	92.7
	Δ	2.8	2.7
	σ	3.4	3.6
Moraxella (groups III+IV+V)	I_φ	76.7	88.2
	$\bar{\Delta}$	5.3	2.4
	σ	6.6	3.3
Groups II+III+IV+V	I_φ	77.4	90.1
	Δ	4.6	3.1
	σ	6.0	4.1
M. osloensis (group IV)	I_φ	82.3	97.0
	$\bar{\Delta}$	4.0	–
	σ	3.8	–
M. phenylpyruvica (group V)	I_φ	80.3	92.0
	Δ	6.1	–
	σ	7.[illegible]	–
M. kingae (group VI)	I_φ	71.6	99.0
	Δ	6.0	–
	σ	6.5	–
M. urethralis (group VII)	I	65.3	–
	Δ	3.4	–
		4.7	–

[1] Mean similarity indices of the groups calculated from the values of Fig. 3 (see also ref. 20)
[2] For definition see ref. 20, Table 1
[3] I: mean transformed Yule correlation coefficient; Δ: mean deviation from the mean; σ: standard deviation.
[4] The discrepancy between these indices and the corresponding indices of ref. 20, Table 4 refers to the effect of the additional *Acinetobacter* fatty acid computed in the present study

hibits the value 93.3 whereas the corresponding value of *Moraxella* is 88.2. Thus, the present results are in accordance with the concept that *Acinetobacter* constitutes one single genus. This is strengthened by the wide variety among strains selected for examination, according to phenotypic properties and DNA homology (Table 1).

The fatty acid composition of *Acinetobacter* appears less homogeneous than usual for a *Neisseriaceae* species. Its mean intragroup similarity index (88.9) is lower than all intra

species indices of neisseriae and moraxellae (Fig 2) The phenogram (Fig 3) indicates three *Acinetobacter* subclusters. One of these, strains nos. 4 5 8, 1 and 2 corresponds fairly closely to the group H1 of *Johnson et al.* (23) and the phenotypic group A of *Baumann et al.* (3) The other two subclusters do not correspond to any particular DNA homology or cultural biochemical group in those studies although most proteolytic strains (nos. 6 7 9 and 12) have a very similar fatty acid composition. Moreover the principal com ponents analysis does not reveal any subdivision (Fig 4) The subclusters might have been formed by coincidence associated with the limited number of strains examined and the results are inconclusive with respect to the possible subdivision of the genus *Acinetobacter*

Except for the oxidase reaction, the phenotypic properties of a few moraxellae resemble those of some of the *Acinetobacter* strains (3 10) and numerical analysis of such properties has not distinguished clearly between the two genera (24 39) Nevertheless, only very low association between *Moraxella* and *Acinetobacter* has been indicated by genetic analysis *Juni* (25) was unable to substantiate any relationship by DNA transformation of non-ribosomal genetic markers. However experi nents on transformation of the ribosomal marker streptomycin resistance, performed with "false neisseriae" (i.e. N catarrhalis) and M osloensis as recipients, have revealed some donor activities of *Acinetobacter* strains (8) Furthermore, recent studies by a similar technique performed with A calcoaceticus BD4 as recipient have shown certain low activities of "false neisseriae" and "classical moraxellae" (*M nonliquefaciens M bovis* and *M lacunata*) donors Marginal affinities to this recipient were also recorded for *M osloensis* but not for donors belonging to *M phenylpyruvica M kingae M urethralis* or "true neisseriae" (unpublished results) Similarly *Johnson et al* (23 found a distant relationship between oxid se positive and negative organisms of these groups by hybridization with ribosom l RNA, whereas only negligible homology was revealed by DNA DNA hybridization.

The present investigation supports the genetic evidence for a separate status of the genus *Acinetobacter* and for a certain relationship between *Acinetobacter* and *Moraxella* plus false neisseriae" Furthermore, it should be noted that the tentatively named *M urethralis* is as different from *Acinetobacter* in terms of fatty acid composition as it is from neisseriae and moraxellae, and that *M kingae* is clearly as distinct from *Acinetobacter* as it is from unequivocal *Moraxella* species (11 20) These findings are also consistent with genetic data (see above)

There is no clear genetic evidence for relatedness between "true neisseriae" and the other genera of *Neisseriaceae* (8, 11 17 and unpublished transformation results) In fact, the similarity in fatty acid pattern of certain true neisseriae" and acinetobacters is one out of very few phenotypic indications of such a relationship

Since the fatty acid composition of *Acinetobacter* has previously been studied only on single isolates (1 12, 22 37 38) and because of the taxonomic confusion within this group of organisms, a comparison between these previous and the present results has been avoided.

As shown in Table 2 both 3-hydroxy- and 2-hydroxy fatty acids are generally present in *Acinetobacter* The 3-hydroxy fatty acids, particularly 3–OH–12·0 and 3–OH–14·0 are common constituents of the endotoxic lipopolysaccharides of Gram-negative bacteria (29) In contrast, 2 hydroxy fatty acids appear less regularly and have previously only been detected within *Pseudomonas* (15) *Azotobacter* (26) *Rhodomicrobium* (31) and a group of motile achromobacters (40) Consequently the presence of the fatty acid 2–OH–12 0 (lacking only in strain ATCC 17985) as well as the unidentified compounds y-1 (present in all strains but ATCC 17906) and y-2 may constitute useful diagnostic markers of *Acinetobacter*

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REFERENCES

1. *Adams, G A. Quadling, C., Yaguchi M & Tornabene T G* The chemical composition of cell-wall lipopolysaccharides from *Moraxella duplex* and *Micrococcus calco-aceticus*. Canad. J. Microbiol. *16* 1–18, 1970.
2. *Anderson, T W* Introduction to Multivariat Statistical Analysis. John Wiley & Sons, Inc. London 1964 p. 272–287
3. *Baumann P., Doudoroff M & Stanier R. Y.* A study of the *Moraxella* group. II Oxidase-negative species (genus *Acinetobacter*) J Bact. 95 1520–1541 1968.
4. *Bergan T* A transformed Yule correlation coefficient employed in numerical grouping procedures on bacteriophage lytic spectra. Acta path. microbiol. scand. Sect. B, *80* 89–100 1972.
5. *Brian B. L. & Gardner E. W* A simple procedure for detecting the presence of cyclopropane fatty acids in bacterial lipids. Appl. Microbiol. *16* 549–552 1968.
6. *Brisou J & Prévot A. R.* Études de la systématique bactérienne X. Révision des espèces réunies dans le genre *A hr mobacter* Ann. Inst. Pasteur *86* 722–728, 1954
7. *Buchanan, R E. & Gibbons N E* Bergey' Manual of Determinative Bacteriology 8th. ed The Williams & Wilkins Co Baltimore 1974
8. *Bøvre K.* Studies on transformation in *Moraxella* and organisms assumed to be related to *Morax lla*. 8. The relative position of some oxidase negative, immotile diplobacilli (*A kromobacter*) in the transformation system. Acta path microbiol. scand 69 109–122, 1967
9. *Bøv e K. Fiandt M & Szybalski, W* DNA base composition of *Neisseria Moraxella* and *Acinetobacter* as determined by measurement of buoyant density in CsCl gradients. Canad. J Microbiol. 15 335–338, 1969
10. *Bøv K. & Henriksen S D* A new *M ax ella* species, *Moraxella osloensis*, and a revised description of *Moraxella nonliq faciens*. Int. J system. Bact. *17* 127–135 1967
11. *Bøvr K Hytt R., Jantzen E & F hol L. O.:* Gas chromatography of bacterial whole cell methanolysates. III Group relations of neisseriae and moraxellae Acta path. microbiol. scand. Sect B, *80* 683 689 1972.
12. *Cho K Y & Salton M R J.:* Fatty acid composition of bacterial membrane and wall lipids. Biochim. Biophys. Acta *116* 73–79 1966.
13. *De Ley J.* DNA base composition and taxonomy of some *Acinetobacter* strains. Antonie van Leeuwenhoek *34:* 109–114 1968.
14. *Frøholm L. O., Jantzen E. Hytta, R & Bøvre K* Gas chromatography of bacterial whole cell methanolysates. II A taxonomic evaluation of the method for species of *Moraxella*. Acta path. microbiol. scand. Sect. B, *80* 672–682, 1972.
15. *Hancock I C Humph eys G O & Meadow P M.* Characterization of the hydroxy acids of *Pseudomonas aeruginosa* 8602. Biochim. Biophys. Acta *202:* 389–391 1970
16. *Hatt H D., Lessel, E F., Clark W A., Davis E E., Jong, S C Zieg, R. G & Alexander M T:* The American Type Culture Collection. Catalogue of Strains, 11th. ed American Type Culture Collection, Rockville, Maryland 1974
17. *Henriksen S D.* *Moraxella Acinetobacter* and the *Mimeae* Bact. Rev *37* 522–561 1973
18. *Jantzen E. Bergan T & Bøvre K* Gas chromatography of bacterial whole cell methanolysates. VI Fatty acid composition of strains within *Micrococcaceae* Acta path. microbiol. scand. Sect. B *82* 785–798, 1974
19. *Jantzen, E., Bryn K & Bøvr K* Gas chromatography of bacterial whole cell methanolysates IV A procedure for fractionation and identification of fatty acids and monosaccharides of cellular structures Acta path. microbiol. scand. Sect. B, *82* 753 766 1974
20. *Jantzen E Bryn K Bergan T & Bøvr K* Gas chromatography of bacterial whole cell methanolysates. V F tty acid composition of neisseriae and moraxellae. Acta path. microbiol. scand. Sect B, *82* 767–779 1974
21. *Jantzen E., F holm, L. O Hytt R & Bøvr K* Gas chromatography of bacterial whole cell methanolysates. I The usefulness of trimethyl- and trifluoroacetyl derivates for strain and species characterization. Acta path. microbiol. scand. Sect. B, *80* 660–671 1972.
22. *Jauch R., Schiele U & Lynen F* Die höheren Fettsäuren in *A kromobacter*. Arch. Microbiol. *72* 186–198, 1970
23. *Johnson J L., Anderson R. S & O del E J* Nucleic acid homologies among oxidase negative *Mor xella* species. J Bact *101* 568 573 1970.
24. *J hnson R. & Sneath P H A* Taxonomy of *Bordetella* and related organisms of the families *Ach omobacteria ea Bruc llaceae* and *Neisseriaceae* Int. J system. Bact *23* 381 404 1973
25. *J el E.* Interspecies transformation of *Acin-*

etobacter Genetic evidence for a ubiquitous genus. J Bact. 112 917–931 1972.

26. *Kaneshiro T & Marr A G* Hydroxy fatty acids of *Azotobacter agilis* Biochim. Biophys. Acta 70 271–277 1963

27 *Lautrop H Bovre K. & Fredriksen W* A *Moraxella*-like microorganism isolated from the genito-urinary tract of man. Acta path. microbiol. scand. Sect. B 78 255–256 1970

28 *Lessel E. F* International Committee on Nomenclature of Bacteria. Subcommittee on the Taxonomy of *Moraxella* and Allied Bacteria. Int. J system. Bact. 21 213–214 1971

29 *Lüderitz O Westphal O., Staub A M & Nikaido H* Isolation and chemical and immunological characterization of bacterial lipopolysaccharides. In Weinbaum, G Kadis, S & Ajl, S J (Eds.) Microbial Toxins. Vol. IV Bacterial Endotoxins. Academic Press, Inc. 1971 p. 145–233

30 *Mannheim W & Stenzel W* Zur Systematik der obligat aeroben gramnegativen Diplobakterien des Menschen. Zbl. Bakt., I Abt. Orig 186 55–83 1962.

31 *Park C E & Berger L. R.* Fatty acids of extractable and bound lipids of *Rhodomicrobium vannielii*. J Bact. 93 230–236 1967

32 *Piéchaud D Piéchaud M & Second L* Variétés protéolytiques de *Moraxella lwoffii* et de *Moraxella glucidolytica (Bacterium anitratum)* Ann. Inst. Pasteur 90 517–522, 1956.

33 *Ryhage R. & Stenhagen E.* Mass spectrometric studies VI Methyl esters of normal chain oxo- hydroxy methoxy and epoxyacids. Ark. Kemi 15 545–569 1960

34 *Samuels S B., Pittman B., Tatum H W & Cherry W B.* Report on a study set of moraxellae and allied bacteria. Int. J system. Bact. 22 19–38, 1972.

35. *Shaw N* Lipid composition as a guide to the classification of bacteria. Advanc. appl. Microbiol. 17 63–108, 1974

36. *Sokal R R. & Michener C. D* A statistical method for evaluating systematic relationships. Kans. Univ Sci. Bull. 38 1409–1438, 1958.

37 *Stenhauer J E. Flentge R. L. & Lechowich, R V.* Lipid patterns of selected microorganisms as determined by gas-liquid chromatography Appl. Microbiol. 15 826–829 1967

38. *Thorne K J I., Thornley M J & Glauert A M* Chemical analysis of the outer membrane and other layers of the cell envelope of *Acinetobacter* sp. J Bact. 116 410–417 1973.

39 *Thornley M J* A taxonomic study of *Acinetobacter* and related genera. J gen. Microbiol. 49 211–257 1967

40 *Yabuuchi E. Yano I., Goto S Tanimura, E., Ito T & Ohyama A* Description of *Achromobacter xylosoxidans* Yabuuchi and Ohyama 1971 Int. J system. Bact. 24 470–477 1974

Acta path. microbiol. scand. Sect. B, 83: 581–589, 1975

THE ULTRASTRUCTURE OF *TRICHOMONAS VAGINALIS* DONNÉ BEFORE AND AFTER TRANSFER FROM VAGINAL SECRETION TO DIAMONDS MEDIUM

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Nielsen, M. H. The ultrastructure of *Trichomonas vaginalis* Donné before and after transfer from vaginal secretion to Diamonds medium. Acta path. microbiol. scand. Sect. B, 83: 581–589, 1975.

Cells of *Trichomonas vaginalis* (*T. vag.*) obtained from five patients with severe trichomoniasis were fixed for electron microscopy directly in the vaginal fluid. Samples of the same material from three of the patients was inoculated into Diamonds medium and propagated for 3–6 weeks. During the first 3–4 weeks *in vitro* the fine structure of all cells was changed. The shape of the nucleus, the size of the Golgi apparatus and adjacent vesicles, the fine structure of the chromatic granules, and the mastigont were all changed when compared to the organelles of the originally isolated cells. In addition the ectoplasm had disappeared, the glycogen granules had become sparse, and the number of free cytoplasmic ribosomes had increased. On the other hand, the large food vacuoles and the cell membranes generally were unchanged. Mycoplasms were observed in the original material and were also seen after more than 6 weeks of propagation, both as free organisms in the culture medium and within the large food vacuoles of *in vitro* cultured cells.

Key words: *Trichomonas vaginalis*; ultrastructure.

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The size and shape of organisms of *Trichomonas vaginalis* Donné (*T. vag.*) present in vaginal secretion is more variable than the size and shape of organisms grown *in vitro*. Major morphological differences which may be present between cells *in vivo* usually disappear after growth for a few weeks *in vitro* (4, 6, 27). *T. vag.* cells grown *in vivo* and *in vitro* also differ with respect to their fine structure (1, 18). Presently, however, it is unknown when the changes occur after cells are transferred from *in vivo* to *in vitro* conditions of growth.

The purpose of the present investigation was to examine the fine structure of *T. vag.* cells present in vaginal secretions and compare their structure with the structure of cells obtained after propagation of samples of the vaginal secretions in Diamonds medium (3).

MATERIAL AND METHODS

In five patients, attending the outpatient clinic of Rudolph Berghs Hospital and the Sonderbro Hospital, Copenhagen, cells of *T vag* were demonstrated in the vaginal secretion both by direct microscopy of wet smears and by cultivation as described previously (20)

Electron microscopy was performed on *T vag* cells obtained directly from the vaginal secretion, and on cells inoculated into Diamonds medium with antibiotics (5) These axenic cultures were maintained at 37 C for 3 to 6 weeks by subcultivation every 48 hours.

Fixation of T vag cells in vaginal fluid Approximately 2 ml of the highly viscous vaginal fluid was removed with a sterile pipette, and mixed with 4 ml 3 per cent glutaraldehyde in 0 1 M cacodylate buffer pH 7.2 After fixation for 30 to 60 minutes at room temperature the cells were collected by centrifugation at 1000 *g* for 10 minutes. The pellet obtained was washed overnight at 4 C in 0.1 M cacodylate buffer pH 7.2 with 0 M sucrose added, and fixed in 1 per cent osmium tetroxide dehydrated in ethanol, followed by 100 per cent epoxypropane embedded in Vestopal W and processed for electron microscopy as previously described (15)

Fixation of T vag cells in "in vitro" culture 24 hour cultures of *T vag* cells in a Diamond medium without agar were centrifuged at approximately 600 *g* The pellets obtained were fixed for 30 to 60 minutes in either 1 5 per cent or 3 per cent glutaraldehyde in 0 1 M cacodylate buffer pH 7 2 with or without 0 2 M sucrose added. They were processed for electron microscopy as described above (15)

Electron microscopy was performed on a Philips model 200 or a Siemens Elmiscope model 1A electron microscope

RESULTS

fter 2–4 weeks of maintenance in Diamonds edium cells of *T vag* presented a shape d fine structure clearly different from the s of the original inoculum Irregularly amoeboid cells (Fig 1 were gradually replaced by rounded, ovoid non-amoeboid cells (Fig 2) Great variations were encountered however from one individual ell to another as some cells retained the m h logy characteristic of cells of the vagi retion for as long as four weeks, while ells not could be found in other cultur after the first week *in vitro*

After cultivation in Diamonds m for 3–4 weeks, cells with a distinct marginal "ectoplasm" (EC, Figs. 1 5) had disappeared. In the vaginal fluid cells with "ectoplasm" usually were attached to desquamated epithelial cells, in the *in vitro* material they were generally attached to other trichomonads. The "ectoplasm" showed the same fine structure both in *T vag* cells maintained *in vitro* and in cells of the vaginal secretions and was identical with that of the "ectoplasm" of *T vag* previously described (18)

After cultivation *in vitro* for 4–6 weeks the irregularly shaped nuclei (N Fig 1) of *T vag* cells had achieved an ovoid shape (N Fig 2) Their contents of chromatinic material (CH, Figs. 1 2) however were largely unchanged.

The contents of the phagosomes or food vacuoles of cells transferred to Diamonds medium had already after a short period of propagation changed probably because bacteria and epithelial cell debris originally present were rapidly digested *in vitro* Therefore small phagosomes with freshly engulfed material were sparse whereas the number of the large relatively empty food vacuoles was unchanged in cells propagated for 4–6 weeks in Diamonds medium. The large food vacuoles were more or less identical to those of the cells of the *in vivo* material with diameters generally between 0 4 and 1 μm.

Mycoplasms were present in the culture medium and in the phagosomes of cells which had been propagated for 4–6 weeks. The majority of the engulfed mycoplasms were located in large food vacuoles (PL Fig 1) and showed no sign of lysis.

Pinocytotic invaginations and vesicles were numerous in *T vag* cells of both the *in vivo* and *in vitro* material.

The Golgi regions of cells from the first 6 weeks of cultivation *in vitro* showed only slight changes in comparison with those of

All illustrations show sections of *T vag* cells embedded in Vestopal-W Sections were post-stained with uranyl and lead salts. For details of preparation see reference (15) Unless otherwise stated the bar on each micrograph represents 0.5 μm.



Fig 1 Part of two *T* *vag* cells from vaginal fluid. The irregularly shaped nucle[illegible] and the irregularly distributed chromatin (CH) [illegible] visible in one cell. CG denotes chromatic g[illegible] large food vacuoles with engulfed mycoplasma. The less dense areas of the cytoplasm [illegible] (PL) the ectoplasm (EC) which in this particular cell does not form [illegible] coherent layer [illegible] phery. Part of epithelial cell cytoplasm (EP) is also seen. Magn 14 500

Fig 2 *T* *vag* cell from culture obtained three weeks after transfer to D[illegible] mond medium. The nucleus (N) and the large nucleolus (NN) are both of regular shape. The chromatin (CH [illegible] regularly distributed. Large food vacuoles (PL) ha[illegible] scanty electron-dense contents. Chromatic granules are marked CG. Magn 14 500

cells from the vaginal secretion. The number of flat saccules was between 11 and 16 in cells isolated from the vaginal fluid, and between 11 and 13 in cells grown *in vitro*. It was apparent that the number of surrounding Golgi vesicles with or without electrondense content, was smaller in cells from the *in vitro* material. The limiting membranes of the Golgi vesicles (GV Fig 3) and saccules were of the unit membrane type and approximately 8 nm wide.

Generally the inner leaflets of these membranes were more intensely stained than the outer. This was especially apparent in saccules located near the cell surface (Arrow Fig 3). Four to eight of the centrally located saccules were separated by a 10 to 11 nm thick layer of electron-dense material (DM Fig 3). This layer was found in more than 50 per cent of the cells independently of whether they were obtained from the *in vivo* or *in vitro* material.

In *T vag* cells from the vaginal fluid an arrangement of 3 or 4 flat "collapsed vesicles" (VE, Figs. 3 4) was present. The "vesicles" were always arranged in parallel layers near the cell membrane. Their "lumina" were obliterated or occupied by electron-dense material. The width of their limiting membrane was approximately 8 nm. These structures have not, to my knowledge been described before and were never seen in the *in vitro* material.

The cell membrane (CM Figs. 3 4) was between 11 and 14 nm thick. The varying thickness was mainly due to varying amounts of a fluffy substance present on the exterior side of the outer leaflet. The inner electron-dense layer generally [illegible] 3 nm the outer 4–7 nm thick. The [illegible] membranes of cells obtained from either [illegible] teral were identical. [illegible] membrane was covered [illegible] coat (16 18) (CC Figs [illegible] at pinocytotic invaginat[illegible] of cells from vaginal secr[illegible] nm wide and that of cells p[illegible] 30–40 nm wide.

The matrix of chromatic gr[illegible] which were obtained from the *in vivo* material occasionally displayed small areas of more electron-dense material as previously described (18). In cells of the *in vivo* material the chromatic granules (CG Figs. 7 9) usually were surrounded by glycogen granules (GL, Figs. 7 9) and only by scattered clusters of ribosomes (RI Figs. 7 9). However in cells propagated in Diamonds medium the chromatic granules were usually surrounded by rather densely packed ribosomes (RI Fig 8) and no glycogen granules were present.

As a whole, glycogen granules were sparse in *T vag* cells which were maintained *in vitro* for more than 2 weeks, but a few cells with an unaltered high glycogen content were still present after 3 weeks of propagation *in vitro*. Glycogen appeared either as the compact granules previously described (18) (CL, Figs. 4 9) or as rosettes (GL, Fig. 7).

These α-granules or rosettes generally measured 50 to 86 nm in diameter whereas the small dense β-granules generally were 3–4 nm wide. An electron-translucent zone

Fig 3 The Golgi apparatus of a *T vag* cell from vaginal fluid. Some dense material (DM) is present between 8 of the flat Golgi saccules. Golgi saccules situated closer to the cell surface show increased electron-density of the inner laminae of their limiting membranes (arrow). Golgi vesicles are marked GV the cell membrane (CM) and 3 collapsed vesicles" (VE). PF denotes parabasal filament. Magn. 87 000 ×

Fig 4 Part of a *T vag* cell from vaginal fluid. Three collapsed vesicles" (VE) are arranged in parallel near the cell membrane. Their limiting membrane is approximately 8 nm wide. The cell membrane (CM) is wider and consists of a wide intensely stained outer leaflet, and a narrower less intensely stained inner leaflet. Note fluffy substance on exterior surface of cell membrane GI denotes glycogen granules. Magn. 91,000 ×

Figs 5 and 6 Filamentous cell coat (CC) on a *T vag* cell obtained from vaginal secretion (Fig 5) and on a cell obtained from a cell culture after [illegible] weeks of *in vitro* propagation (Fig. 6). In Fig 5 glycogen granules (GI) and ectoplasm (EC) are also visible. Magn. Fig 5: 87,000 × & Fig. 6 [illegible] 000

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was seen to surround some glycogen granules (GL, Fig. 7) This zone was at maximum 13 nm wide. It was usually distinct in cells from the vaginal secretions but not in cells of the in vitro material.

After cultivation in vitro the amount of cytoplasmic ribosomes of cells was increased compared to that found in cells from the in vivo material, especially around the chromatic granules (RI, Fig. 8)

The presence of 2 axostyles in one cell, the lack of protrusion of the caudal part of the axostyle, and the rare intracytoplasmic flagellar axonemes were found in cells from the vaginal fluid only

DISCUSSION

The large food vacuoles in cells of *T vag* were not changed with respect to size and number when cells were transfered from vaginal secretion to the Diamond medium, even though the cells were thereby deprived of corpuscular material such as bacteria and epithelial cell debris. The large food vacuoles, therefore, possibly have other functions than acting as plain phagolysosomes (16, 17) or as reservoirs for partly digested material Their large size and low content of electron-dense material may indicate that they—in analogy with the contractile vacuoles of some protozoa (9)—have additional osmoregulatory functions. The rapid formation and disappearance of vacuoles occasionally observed in living *T vag* cells (23) support this assumption.

The present investig tion demonstrates that bacteria of the aginal fluid, but not mycoplasms, are lysed when engulfed by *T vag* cells. Similar findings were published previously (18) How long mycoplasms may stay morphologically undamaged in the large food vacuoles, and whether they are still viable under these conditions is presently unknown.

The dimensions of the limiting membranes of the "collapsed vesicles" are identical to those of the Golgi esicles or the smooth endoplasmic reticulum. Whether they originate from any of these structures is difficult to say Any functional significance of their arrangement in parallel layers is also obscure.

An abnormal fine structure of mastigont organelles was found previously in *T vag* cells from experimental or natural infections (1 18) in cells of *Tetratrichomonas limacis* from in vivo material (21) and in pseudocysts formed in vitro of cells of *Trichomitus batrachorum* (13) In the present study a rapid normalization of the mastigont fine structure occurred after cells were transferred to Diamonds medium. This probably indicates that the abnormal morphology was due to suboptimal nutritional conditions in vivo The abnormal mastigonts therefore may be considered as "degenerative forms" like the mastigonts of trichomonad pseudocysts in general (14 22)

The glycogen content of extracellular parasitic protozoa usually drops when cells are transferred from the natural infection to artificial culture media. The growth rate of freshly isolated cells also changes and is usually increased when cells are maintained in vitro for some time (7 10) The present study demonstrates both types of changes in *T vag* cells and usually they occurred simultaneously The drop in cellular glycogen content therefore may be secondary to the increased rate of cell growth (2) For different *T vag* cells this change of morphology occurred at various times during the f rst 3 eeks after transfer to Diamonds medium This variation indicates that the change of morphology was dependent not only on environmental factors but also on some physiological factors of the particular cells as well.

The virulence and infectivity of many parasitic protozoa is changed when they are cultivated in vitro for some time (7) Among trichomonad species this has been recorded in *Trichomonas gallina* (*T gal.*) and *T vag* (5 7 11 24 25) The attenuation of pathogenicity of *T gal.* was enhanced by the presence of antibiotics, especially streptomycin, but impeded if the parasites were caused to divide less frequently during a given period

of time and completely prevented if the trichomonads were maintained in tissue cultures (8 24) The Antibiotics were supposed to act directly on the high molecular RNA of the trichomonad cells (24) In light of the present findings the activity of antibiotics may also be due to their effect on concomitant microorganisms. In this connection it should be mentioned that bacteria previously have been suspected to be an environmental factor enhancing the virulence of trichomonads (5)

The virulence of the *T vag* isolates of the present study was high because they were isolated from patients with severe trichomoniasis (10) Probably it was also unaltered during growth *in vitro* because it has been demonstrated that an attenuation of the *T vag* virulence *in vitro* needs several months to be established (11 12) The present study therefore gives no direct information on morphological differences between virulent and avirulent *T vag* cells. This can be obtained, however if the fine structure of the five presently and four previously studied *T vag* [illegible] (18) is compared with the fine struc[illegible] a strain which was maintained by sub[illegible] in the Diamond medium for more [illegible] year The *T vag* cells obtained direct[illegible] from vaginal fluid all had cell membranes which showed a wider outer leaflet and a thinner inner leaflet, whereas the cell membranes of cells propagated *in vitro* for 1 year displayed two almost equally wide electron dense layers (19). It is tempting to explain this difference by the presence of a 1–4 nm wide amorphous cell coat on the cell surface of virulent *T vag* cells. This explanation may obtain some support from the fact that a proteinaceous cell coat of similar appearance is present on other virulent parasitic protozoa (26) It must be emphasized however that this cell coat should be distinguished from the filamentous cell coat previously described for cells of *T vag* (16 18) The latter type of coat consists of minute filaments and only covers a minor part of the cell surface.

REFERENCES

1 *Brugerolle G., Gobert J G & Savel J* Étude ultrastructurale des lésions viscérales provoquées par l'injection intra-péritonéale de *Trichomonas vaginalis* chez la souris. Ann. Parasit. (Paris) *49* 301–318, 1974

2. *Daly J J Matthews H M., Koch W G. & Bost J W.*. The effect of different carbohydrates on the glycogen content and growth of *Trichomonas gallinae* Comp. Biochem Physiol. *48 B* 651–659 1974

3 *Diamond L. S* The establishment of various trichomonads of animal and man in axenic cultures. J Parasit. 43 488–498, 1957

4 *Glebski J* Morphological variability of *Trichomonas vaginalis* in biological material and in culture. Wiad. Parazyt. *15* 245–249 1969.

5 *Honigberg, B. M.*. Comparative pathogenicity of *Trichomonas vaginalis* and *Trichomonas gallinae* to mice I Gross pathology quantitative evaluation of virulence and some factors affecting pathogenicity J Parasit. *47*: 545–571 1961

6. *Honigberg, B. M & King, V M* Structure of *Trichomonas vaginalis* Donné J Parasit. *50* 345–364 1964

7 *Honigberg B M.*. Some recent advances in our understanding of parasitic protozoa. J. Protozool. *15* 223–230 1968.

8. *Honigberg B M., Stabler R M Livingston M C & Kulda J* Further observations on the effect of various laboratory [illegible] tres on the virulence of *Trich*[illegible]

chomonads. Das Deutsche Gesundheitswesen 23 2001–2005 1968.

15 *Nielsen M H., Ludvik, J & Nielsen R.* On the ultrastructure of *Trichomonas vaginalis* Donné. J Microscopie 5 229–250 1966.

16. *Nielsen, M H.:* Phagocytosis by *Trichomonas vaginalis* Donné. Substructural and electron-histochemical observations. Sept. Congr Int. Micr Electr 2: 389–390 1970

17 *Nielsen M H.,* Fine structural localization of nucleoside triphosphatase and acid phosphatase activity in *Trichomonas vaginalis* Donné. Cell Tiss. Res. *151:* 269–280, 1974

18. *Nielsen M H & Nielsen R.,* Electron microscopy of *Trichomonas vaginalis* Donné. Interaction with vaginal epithelium in human trichomoniasis. Acta path. microbiol. scand. Sect. B, *83* 305–320 1975

19. *Nielsen, M H.,* Unpublished results.

20. *Nielsen R.: Trichomonas vaginalis* II. Laboratory investigations in trichomoniasis. Brit. J vener dis. *49* 531–535 1973

21 *Salenddin A S M :* Fine structure of *Tetratrichomonas limacis* (Dujardin) Canad. J Zool. *50:* 695–701 1972.

22 *Samuels R.,* Studies on *Tritrichomonas batrachorum I* The trophic organism. J Protozool. 4 110–118, 1957

23 *Smith B. F :* Fine structure of *Trichomonas vaginalis*. Exptl. Parasit. *19* 52–63 1966.

24 *Stabler R. M., Honigberg, B. M & King, V M :* Effect of certain laboratory procedures on virulence of the Jones' Barn strain of *Trichomonas gallinae* for pigeons. J Parasit. *50:* 36–41 1964

25 *Teras J :* On the varieties of *Trichomonas vaginalis*. Progress Protozool. 197–198, 1965 Internat. Congr Series No 91 Excerpta Medica Foundation Amsterdam, New York, London.

26. *Vickerman, K.,* On the surface coat and flagellar adhesion in trypanosomes. J Cell sci. 5 163–193 1969

27 *Winston R. M L.* The relation between size and pathogenicity of *Trichomonas vaginalis*. J Obstet. Gynec. Brit. Com. *81:* 399–404 1974

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THE SEROLOGY OF *BORDETELLA BRONCHISEPTICA* ISOLATED FROM PIGS COMPARED WITH STRAINS FROM OTHER ANIMAL SPECIES

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Pedersen, K. B. The *serology of Bordetella bronchiseptica isolated from pigs compared* with strains from other animal species. Acta path. microbiol. scand. Sect. B 83 590–594 1975.

The serology of freshly isolated strains of *Bordetella bronchiseptica* from pigs and some other animal species was studied by the agglutination and agglutinin absorption tests. Differentiation was made between heat stable O-antigens and heat labile K-antigens. All the strains examined contained a common O-antigen (1) In addition, three heat stable antigens were detected. The heat labile antigens of freshly isolated strains were found to be very different. The major antigens 2 and 3 were not found in combination. A common weak K-antigen (1) was found in all strains. The pig strains were found to be serologically identical (O 1 2 K 1 2) The same serotype was isolated from a rabbit. Another rabbit strain was found to be similar to a strain isolated from an SPF rat (O 1 3; K 1 3 4) The antigenic pattern found in two cat strains was O 1 3 K 1 3 Typing of freshly isolated strains from pigs with monospecific factor sera confirmed that all the strains so far examined were serologically identical. This uniform serology hampers epidemiological studies of porcine bordetellosis by antigenic analysis. The occurrence of serologically different serotypes of *B. bronchiseptica* is of significance in the diagnostic bacteriology since the final diagnosis must be confirmed by serological test.

Key words *Bordetella bronchiseptica* serology pigs other animal species.

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Bordetella bronchiseptica is common in the respiratory tract of several animal species. Great interest in the occurrence of this organism in swine arose from the demonstration of it as an aetiological factor in atrophic rhinitis (9) The epidemiology of bordetellosis in swine is incompletely known. One important question is whether swine are infected from other animal species and, if so, to what extent.

The purpose of the present work was to compare the antigenic structure of porcine strains of *B. bronchiseptica* with that of strains from other animal species, and thereby possibly further elucidate the epidemiology of porcine bordetellosis.

The serological methods used in the present study are based on the principles of *Kauffmann* (3) which were also used by *Andersen* (1) in her studies on the serology of the genus *Bordetella* Accordingly a differ

TABLE 1 *Cross Agglutination Tests between Unheated and Heated Antigens and their Homologous Antisera*

Antigen	Strain	Antiserum							
		Merthiolate-killed				120° C-killed			
		4424	835	R 1	M 1	4424	835	R 1	M 1
Merthiolate-killed	4424	2560	40	40	2560	0	10	20	40
	835	40	2560	2560	20	0	10	20	40
	R 1	40	2560	2560	40	0	40	40	40
	M 1	2560	40	80	2560	0	10	20	80
120° C-killed	4424	40	10	20	20	160	640	160	320
	835	40	20	20	20	160	640	320	320
	R 1	40	20	20	20	160	640	320	320
	M 1	40	20	20	20	160	640	320	320

entiation will be made between heat labile K-antigens and heat stable O-antigens.

MATERIALS AND METHODS

Bordetella strains A total of 11 freshly isolated strains were used. Five strains (73 2568, 3531 4389 4424) originated from pigs with pneumonia or atrophic rhinitis. Two of the pigs were SPF Two strains (835 887) were isolated from cats, and tw (361 1024) from rabbits with bronchopneumonia. One strain (M 1) was isolated from a mouse caught on premises housing an SPF swine herd, and one from an SPF rat (R-1)

Antigens The organisms were grown on 5 per cent bovine blood agar medium at 37 C for 24 hours. The antigen was harvested in phosphate buffered saline (pH 7.2) and adjusted to contain approximately 10^{10} living cells per ml

Antisera. For each strain, rabbit antiserum against merthiolate-killed whole organisms and against organisms heated to 120° C for 60 minutes was produced. Before immunization, the rabbits were found free from agglutinating serum antibodies against *B. bronchiseptica*. The rabbits were immunized by intravenous injection of 0.5 1.0 1.5 and 2.0 ml antigen at intervals of 3–4 days. The rabbits were bled 6 days after the last injection.

Agglutination The agglutination test was carried out in tubes with two fold dilutions of serum (0.4 ml) and equal parts of antigen of the same density as that used for immunization. The mixture was incubated at 37 C for one hour and subsequently kept at room temperature. Tests with whole bacteria were read after 24 hours and tests with 120° C heated culture after 48 hours. The titres given in the tables refer to the serum dilution before addition of antigen.

Absorption Dense suspensions of cell were used in quantities depending upon the amount of agglutinins to be removed. The absorption was performed at 37 C for one hour Subsequently the suspension was centrifuged and the supernatant tested for agglutinins against the absorbing antigen. In cases where the first absorption failed to remove all the aggl tinins the process was repeated

RESULTS

Only strains each representing a separate antigenic structure are shown in the tables.

Table 1 shows cross-agglutination tests with merthiolate-killed antigen and heated antigen against the corresponding antisera. It appears that merthiolate killed whole bacteria were agglutinated to high titres by homologous antisera whereas the same sera gave low titres against 120 C treated homologous antigens. On the other hand, sera produced by 120 C treated culture agglutinated the corresponding antigens, but gave low or no titres against unheated antigen. *B. bronchiseptica* thus contains an antigen which makes the organism almost O-inagglutinable. The O-inagglutinability can be removed by heat.

Heat stable antigens Agglutinin absorption tests indicated that all the strains contained a common heat stable O-antigen (Table 2) In addition, it was possible to define three further antigens. All porcine strains were found to be identical (O 1 2) The same serotype was isolated from a rabbit (1024) A strain isolated from a rabbit raised at the

TABLE 2 *Cross Agglutination Test of S... against Heated Antigens Cross Absorbed with Heated Antigens (O-antigens)*

Antigen	Strain	Antiserum 4424				Antiserum 835				Antiserum M 1			
		Non abs	Absorbed with			Non abs.	Absorbed with			Non abs.	Absorbed with		
			4424	835	M 1		4424	835	M 1		4424	835	M 1
120 C-killed	4424	160	0	80	0	640	0	0	0	160	0	80	0
	835	160	0	0	0	640	320	0	320	320	0	0	0
	M 1	160	0	80	0	640	0	0	0	160	160	160	0

TABLE 3 *Cross Agglutination Tests of Sera against Unheated Antigens Absorbed with Heated Homologous Antigens and then Cross Absorbed with Unheated Antigens (K-antigens)*

Antigen	Strain	Antiserum 4424				Antiserum 835				Antiserum R 1			
		K-titre	Absorbed with			K titre	Absorbed with			K-titre	Absorbed with		
			4424	835	R 1		4424	835	R 1		4424	835	R 1
Merthiolate-killed	4424	2560	0	1280	1280	80	0	0	0	40	0	0	0
	835	0	0	0	0	1280	320	0	0	40	640	0	0
	R 1	40	0	0	0	1280	320	0	0	2560	640	160	0

Titre after absorption of the antiserum with heated homologous antigen.

TABLE 4 *Absorptions for the Preparation of Monospecific Factorsera to Heat Stable and Heat Labile Antigens*

	Antiserum	Culture used for absorption	Factor left in serum after absorption
120° O-serum	4424 (1 2)	835 (1 3)	O 2
	835 (1 3)	4424 (1 2)	O 3
	M 1 (1 2,4)	4424 (1 2)	O 4
K-serum	4424 (1 2)	835 (1 3)	K 2
	835 (1 3)	4424 (1 2)	K 3
	R 1 (1 2,4)	4424 (1 2)	K 4

present institute (361) was found to be identical (O 1 3) with the SPF rat strain and the two feline strains examined. The only strain possessing antigen factor 4 (M 1) was isolated from a mouse caught on the premises housing the SPF swine herd where the typical porcine serotype had been isolated from the pigs.

Heat labile antigens Based on the results of the agglutinin absorption tests it was possible to establish four heat labile K-antigens (Table 3) The strains were found to be serologically very heterogenous. All shared a common factor 1 antigen. Each strain was found to possess either a well-developed factor 2 or factor 3 antigen. These antigens were not found in combination. The antigen factor 4 was found in two strains only (R 1 361) and was rather weak. All strains isolated from pigs were identical (K 1 2) and one rabbit strain (1024) had a similar antigenic structure. The bordetellae from two cats had the formula K 1 3

Agglutination of Freshly Isolated B. bronchiseptica *Strains in Monofactorial Sera*

Sera monospecific for heat stable and heat labile antigens were prepared by absorptions as shown in Table 4

A total of 80 freshly isolated strains from pigs with atrophic rhinitis or pneumonia were agglutinated with these sera. The bacteria were isolated from pigs originating from herds scattered throughout the country The antigens were prepared from the first or second 24 hour subculture on 5 per cent bovine blood agar plates and suspended in phosphate buffered saline to a density of 10 organisms per ml. To half of this suspension, merthiolate was added to a concentration of 1:5000 while the other half was heated to 120 C for 60 min. The *agglutination* tests revealed that all the porcine strains so far examined contained the same antigenic structure (O 1 2 K 1 2) An American* and a Dutch* porcine strain of *B bronchiseptica* were shown to be serologically identical with the Danish pig strains.

All the *Bordetella* strains currently isolated from rabbits belonging to the present institute were found to be antigenically identical (O 1 3 K 1 3 4) Isolates from guinea pigs with pneumonia from our laboratory were also of this serotype.

Experimental Inoculation of Pigs with the R 1 Strain of B. bronchiseptica

Two 6-week-old pigs originating from an SPF herd free from *B. bronchiseptica* were inoculated intranasally with the SPF rat strain (R-1) On re-isolation from the pigs, the bacterium was found to have retained its antigenic patern (O 1 3 K 1 3 4) during the colonization of the mucous membranes of the pigs.

* These strains were kindly supplied to this institute by Dr *D L. Harris* Iowa State University Ames, U.S.A. and D *J P W M Akkermans* Central Veterinary Institute, Rotterdam, Holland.

TABI F 2. C ss Agglut nation T st of S a gainst H ated Antigen C oss Absorbed with H ated Antigens (O-antigens)

Antigen	St a n	Antiserum 4424				Antiserum 835				Antiserum M 1			
		Non abs	Absorbed with			Non abs.	Absorbed w th			Non abs.	Absorbed with		
			44 4	835	M 1		4424	835	M 1		4424	835	M 1
120 (I I	44 4	160	0	80	0	640	0	0	0	160	0	80	0
	835	160	0	0	0	640	320	0	320	320	0	0	0
	M 1	160	0	80	0	640	0	0	0	160	160	160	0

TABLL 3 C Agglutination T t of Sera against Unheated Antigens Ab orbed with Heated Homologous Antigens and th n Cross Absorbed with Unheated Antigens (K-antigens)

Antigen	Strain	Antiserum 4424				Antiserum 835				Antiserum R 1			
		K titre	Absorbed with			K-titre	Absorbed with			K titre	Absorbed with		
			4424	835	R 1		4424	835	R 1		4424	835	R 1
Merthiolate-killed	4424	2560	0	1280	1280	80	0	0	0	40	0	0	0
	835	20	0	0	0	1280	320	0	0	40	640	0	0
	R 1	40	0	0	0	1280	320	0	0	2560	640	160	0

Titre after absorption of the antiserum with heated homologous antigen.

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ESCHERICHIA COLI O H SEROTYPES ISOLATED FROM HUMAN BLOOD

Prevalence of the K1 Antigen with Technical Details of O and H Antigenic Determination

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Ørskov F & Ørskov I *Escherichia coli* O:H serotypes isolated from human blood. Prevalence of the K1 antigen with technical details of O and H antigenic determination. Acta path. microbiol. scand. Sect. B, 83: 595–600 1975

Escherichia coli strains isolated from human blood obtained from 539 different patients all over Denmark were examined serologically for O and H antigens. 425 strains could be O grouped with O sera 1 to 150. 90 strains were spontaneously agglutinable. Using O sera to the ten most frequent O groups 2, 4 6, 75 9 8, 18, 7 22 and 1 57 per cent of all strains could be grouped. Using sera corresponding to the ten most frequent O and the ten most frequent H sera, it was possible t O H type 68 per cent of all O groupable strains. The K1 antigen was detected in 18 per cent of the strains.

Key words *Escherichia coli* O.H serotypes K1 antigen human blood.

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Since the early days of *Escherichia coli* serotyping many papers have been published on the prevalence of different antigenic groups in extraintestinal diseases in man and in animals (2, 3 8)

Differences in serogroup prevalence were noted between isolates from different animals, from different pathological conditions, from different age groups and from different geographical areas

This is not the place to evaluate or review this quite inhomogeneous material, inhomogeneous to a large extent because of differing criteria used for isolation and differences in methods of examination.

The present material of *E. coli* strains isolated from blood from patients with suspected septicaemia is thus one further addition to the accumulated inhomogeneous information mentioned above. However it constitutes a large material from a rarely examined group of patients, which is quite extensively examined from a bacteriological point of view since it has been examined in practically all existing *E. coli* O and H antisera. Because of the special interest which the polysaccharide *E. coli* K1 antigen has got

TABLE 1 *O Serum Pools for O Antigen Determination of* E. coli

Pool	O antiserum												
A	1	2	50	53	74	149							
B	3	25	38	115									
C	4	16	18ab	18ac	19								
D	5	7	39	65	70	71	114	116					
E	6	9	30	55	57								
F	8	46	60	75	93								
G	17	44	77	106	111	113							
H	23	26	6	68	73	102							
I	12	15	40	78	87	92	96						
J	13	21	22	3	34	83	85	140					
K	14	20	107	117	118	123	138	148					
L	86	88	90	127	128	141							
M	10	11	24	27	28	29	33	36	37	41	56		
N	42	43	45	48	49	51	52	54	58	59	61		
O	63	64	66	69	76	79	80	81	82	112ab	112ac	144	150
P	84	89	91	95	97	98	99	100	101	103	104	105	
Q	108	109	110	118	120	121	124	125ab	125ac	126	130	131	
R	132	134	136	137	142	143	145	146					
S	35	129	133	135	139	147							
T	151	152	153	154	155	156	157						
U	158	159	160	161	162	163							

Single sera diluted 1:200 (or 1 100)
Pools T and U were added after the completion of this paper and the strains described in the paper were therefore not examined in the sera found in these pools.

recently as a virulence antigen in connection with neonatal meningitis, the prevalence of its K antigen has also been examined.

MATERIAL

...ing the period Nov 1964 to June 1966 539 Escherichia coli strains from the same number of patients were isolated in the Diagnostic Department, Statens Seruminstit t, Copenhagen. The blood specimens were received as "Venule"® from many hospitals all over Denmark N detailed clinical information was available but from the accompanying slips t can be concluded that most of the patients had clinical signs of septicaemia.

The biochemical examinations, which led to the *E. coli* diagnosis, were carried out in the Diagnostic Department, and w re most indebted to Dr *Hans Lautrop* for collecting and examining the strains.

METHODS

All strains belonged to *E. coli* as defined in the 8th Edition of *Bergey's* Manual (1) The analyses of O and H antigens were carried out by the bacterial agglutination technique according to principles used in the WHO Collaborative Centre for Reference and Research on Escherichia. These methods were developed during the course of many years, and are based on principles laid down by *Kauffmann* and coworkers (4) A detailed description has, however not been published for several years and despite the fact that these procedures are constantly undergoing changes, we feel that the time has come to publish a more detailed account.

O antigen determination Smooth colonies were inoculated into ox-heart infusion broth produced at Statens Seruminstitut, incubated overnight at 37 C, followed by heat treatment at 100° C for 1 hour Formalin 0.5 per cent was added for preservation. Such cultures keep well for weeks (and months) at 4 C. The first step in the determination, *i.e* agglutination examination in 21 O serum pools, was carried out in perspex plates containing 8 × 10 round-bottomed wells with a diameter of 15 mm. The components of the single pools were selected primarily to keep as many of the known interrelated cross-reactions confined to the same pools (Table 1) In this first step serum dilution of the single serum in a pool was 1:200. To each well in the perspex plate three drops of serum dilution and three drops of the unknown culture were added The filled trays were shaken, placed in a

TABLE … *H Serum Pools f r H Determination of* E. coli

Pool	H antiserum													
A	1	2	3	4	12	17								
B	5	6	7	8	11	21								
C	9	10	14	15	16	51	52							
D	18	19	20	23	24	25	53	56						
E	26	27	28	30	31	32	45	48	49	54	55			
F	29	33	34	35	36	37	38	39	40	41	42	43	44	46	47

The strains presented in this paper were only examined in H sera 1 to 50

plastic bag with filter paper between the trays to avoid condensation and placed in an oven at 50° C overnight. The following day the plates were read using indirect light against a black background without previous shaking. Positive agglutination reactions were pursued to the single O sera (diluted 1 10) of the pools in question. If a culture was completely negativ in the 1 200 pools, the examination was repeated in pools diluted 1:100. If also negative here, a new overnight broth culture was produced, heated at 120° C for 2 hours and examined in the pooled O sera. In many cases the end result of the above examination was *reactions in two or more different* O sera. At our disposal were about 350 cross-absorbed O standard sera and examination in such sera (factor sera) made it most often possible to allocate an unknown *E. coli* reacting in several O sera to one of the O groups in question. Occasionally this was not possible and in these cases the different O groups to which the strain was related has been recorded. Finally all strains were titrated in two-fold dilutions in the O serum, or O sera, in question, and if the titre was 640 or higher the strain was assigned to that group. W realize that several of the strains examined are not serologically O identical with the O antigen of the respective O antigen test strain. A complete identity examination would demand production of O sera with the examined strains followed by cross-absorption experiments—a venture which is impossible, of course, in an investigation like the present. The O test sera used had homologous titre ≧1280.

Some strains were autoagglutinable after boiling and were not investigated further.

H antigen determination. Motility was examined in tubes with semifluid agar (4). The tubes were inoculated by means of a straight wire and incubated at 37 C overnight. Frequently the boundary between growth and no growth had moved to the bottom, *or near to it, during that period.* With a Pasteur pipette … to 3 cm long cylinder of semifluid culture was retracted from an area not too far from that boundary and two or three drops of this culture were transferred to a broth containing tube without dipping the pipette into the broth. Great care was taken to avoid admixture of culture from outside of the pipette, which to *a large extent consisted of surface growth from* the tube with semifluid agar. The inoculated broth was then incubated at 37 C in a rotating drum set on an angle of 45 C for 4 to 5 hours. At this time the culture most often consisted of actively motile organisms suitable for H determination. Only rarely was it necessary to use two or more passages in semifluid agar. U tubes were not used, as many years' experience have taught us that they have no advantages compared with the ordinary semifluid agar tubes, when used as described above. *Formalin 0.5 per cent was added to the* actively motile broth cultures for preservation.

Strains immotile at 37 C were left at room temperature for 2 more days, a procedure which occasionally provides one with a motile culture. Recently the procedure for such strains has been changed and they are now grown both in semifluid agar and in final broth culture at 30° C.

The H standard sera were pooled in six pools which would bring as many cross-reacting sera together in the same pool as possible (Table 2).

The single sera in the pools were used in dilution 1:100 before addition of culture. H agglutination was carried out in 10 × 70 mm tubes. The first two steps: 1) determination of pool and 2) determination of the single H serum in question were carried out by mixing three drops of culture with three drops of serum dilution. The final titration was carried out as *ordinary two-fold* tube titrations. Some cross-reactions exist between H test antigens: the relevant cross-absorbed H factor sera were available t the Escherichia Reference Centre to make it possible to obtain a definit H type in cases of such cross-reactions. Agglutination racks were incubated in waterbath for 2 hours at 50° C.

K1 determination. In order to get an impression of the prevalence of the K1 antigen in the present material, 261 randomly chosen strains were examined in a K1 specific serum. The strains

flora in such a way that the prevalence of a restricted number of serotypes is amplified during the selection which proceeds to invasion of the blood stream.

As most of these frequent serotypes isolated from the blood belong to O groups which ordinarily have acidic polysaccharide capsules (5) the idea suggests itself that the capsule is a necessary constituent of these cells which are able to invade the blood stream.

Serotyping of *E. coli* except for the enteropathogenic types, has not been widely used as a tool in clinical microbiology. One reason for this might be the frightening number of antigens involved.

It is apparent from the present data that with a rather limited number of test sera it will be possible to type a considerable number of strains. For complete serotyping K typing should be included. At present, determination of K antigens following the accepted procedures is not meaningful (5) but useful principles for determination of *E. coli* polysaccharide K antigens are on the way (7). K typing of the strains involved in this series has not yet been carried out, with the exception of the K1 antigen which is a very frequent K polysaccharide antigen and recently has got a special interest. The hitherto used method for detection of the K1 antigen have not been satisfactory but the use of cross-reacting sera produced with *Neisseria meningitidis* type B together with specially produced K1 sera has made it possible to type this speciel K antigen.

Serotyping of *E. coli* strains will probably also in the future be a matter for specialized laboratories, but with the increasing importance of *E. coli* in many inflammatory conditions it might be pointed out that some useful grouping of *E. coli* is possible with a limited number of sera.

REFERENCES

1. *Bergey's* Manual of Determinative Bacteriology. 8th Edit., Williams and Wilkins Co., Baltimore 1974. p. 293–296.
2. *Ewing, W. H. & Davis, B. R.*: The O antigen groups of *Escherichia coli* from various sources. CDC Publication 1961. p. 1–21.
3. *Grüneberg, R. N., Leigh, D. A. & Brumfitt, W.*: In O'Grady, F. & Brumfitt, W. (Eds.) Urinary Tract Infection, Oxford University Press 1968.
4. *Kauffmann, F.*: The bacteriology of *Enterobacteriaceae*. Munksgaard, Copenhagen 1966, p. 19–48.
5. *Ørskov, F., Ørskov, I., Jann, B. & Jann, K.*: Immunoelectrophoretic patterns of extracts from all *Escherichia coli* O and K antigen test strains. Correlation with pathogenicity. Acta path. microbiol. scand. Sect. B, 79: 142–152, 1971.
6. *Robbins, J. B., McCracken, G., Gotschlich, E., Ørskov, F., Ørskov, I. & Granberg, C.*: *Escherichia coli* capsular polysaccharide associated with neonatal meningitis. New Engl. J. Med. *290*: 1216–1220, 1974.
7. *Semjen, G., Ørskov, I. & Ørskov, F.*: Acidic polysaccharide K antigens of *Escherichia coli* determined by counterimmunoelectrophoresis. Unpublished work.
8. *Sojka, W. J.*: *Escherichia coli* in animals. Commonwealth Bureaux, Farnham Royal, 1965. p. 65–183.

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